Dissertation

# Role of Cytokines for NK cell Competence and Differentiation

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# 1 Introduction

## 1.1 The Immune System – Innate and Adaptive Immunity

In order to protect from pathogenic infections all higher eukaryotic species developed a complex system of defence mechanisms entirely designated as immune system. The defence against various pathogens like bacteria, fungi, viruses and parasites as well as the recognition and elimination of virally transformed or tumour cells is exerted by distinct specialised immune cells originating from the bone marrow and circulating in the blood. The immune system can be divided into two arms; the innate and the adaptive system. Both systems interact and complement each other to provide the best possible protection of the body against various pathogens and infectious agents.

### *1.1.1 The innate immune system*

In case of an infection by a pathogen, the different components of the innate immune system become activated immediately, representing therefore the first line of defence. The innate immune system offers a first barrier against pathogen penetration as well as a general defence that is only to certain extend antigen-specific. Physical barriers (skin and mucosal membranes), which prevent the infection by a pathogen, are combined with a set of cellular mechanisms and soluble factors that are intended to eliminate a pathogen once the infection occurred. The most important cells responding to an infection are phagocytic white blood cells like macrophages and neutrophils, able to ingest and kill microbes by producing toxic chemicals and degradative enzymes, and Natural Killer (NK) cells, which mediate lysis of target cells. During the early phase of the innate immune response, both cell types produce cytokines, which cause local inflammation and activate the adaptive immune system. NK cells, which are crucial for early defence against infections and tumour surveillance, represent a connecting cell type between innate and adaptive immune system and will be described in more detail in the next paragraphs.

In order to be alerted, the innate immune system displays a set of receptors, which senses the presence of a pathogen or of danger. These activating receptors are germline-encoded and thus can only detect general patterns, so called pathogen-associated molecular patterns (PAMP). PAMPs are molecules selectively expressed by the pathogen, such as lipopolysaccaride (LPS), flagellin or peptidoglycan. They are recognized by specialized receptors like Toll-like receptors (TLR) expressed on dendritic cells (DCs) and macrophages, and activating receptors expressed on NK cells (1). The binding of PAMP to cells of the innate immune system results in killing of the pathogens and secretion of pro-inflammatory cytokines. The innate immune system acts within minutes to hours after an infection and many of the effector and costimulatory molecules generated during this early phase of the immune response play an important role for the slower developing adaptive response.

### *1.1.2 The adaptive immune system*

Although 90% of infections are eliminated by mechanisms of the innate immune system, some pathogens escape these defences and the adaptive immune system needs to be activated. Soluble factors belonging to the complement system and chemokines and cytokines secreted by innate immune cells induce the recruitment of lymphocytes and the activation of the adaptive immune system. Adaptive immunity is mainly exerted by two types of lymphocytes, namely T cells and B cells. Different from innate immune cells, T and B cells express antigen specific receptors (T cell receptor, TCR and B cell receptor, BCR) which undergo genetic recombination in somatic cells. This process provides with a highly diverse repertoire of receptors able to recognize plenty different pathogen-derived antigens. In contrast to innate immunity, adaptive immunity not only contributes to pathogen clearance but also is essential for the formation of an immunological memory – a feature leading to fast acting responses in case of reinfection. The protection of the extracellular fluids and spaces is mediated by the humoral immune response, in which antibodies produced by B cells bind to extracellular pathogens and toxins. Activation of naïve B cells is triggered by binding of the BCR to its specific antigen and it usually requires the help of T cells. Following antigen binding to the BCR, B cells become activated and differentiate into antibody secreting plasma cells. The secreted antibodies bind specifically to the antigen on the pathogen surface, subsequently leading to complement activation and phagocytosis of the pathogen. Also NK cells recognize target cells coated with antibodies, which leads to lysis of the target cell by so-called antibody dependent cellular cytotoxicity (ADCC).

Different from B cells, T cells are not able to recognize the pathogen directly, but need the help of a professional antigen-presenting cell (APC) such as a DC. DCs which have been activated by pathogen-derived PAMP degrade the pathogen in a specialized cellular compartment named proteasome and present the antigens as small peptides on major histocompatibility complex (MHC) molecules expressed on their surface. The peptide-MHC complex presented by DCs is recognized by the specific TCR expressed on a T cell, leading to T cell activation and clonal expansion. Antigen presentation and activation of naive T cells occur in secondary lymphoid organs where DCs migrate to, after they have encountered the pathogen in the peripheral tissue. Among T cells, two populations can be distinguished, $CD4^+$ T helper ($T_H$) cells and $CD8^+$ cytotoxic T cells (CTL). The latter recognize peptides derived from intracellular pathogens, like viruses, protozoas and some bacteria, that are presented via ubiquitously expressed MHC class I molecules. Subsequent, $CD8^+$ T cell activation and release of cytotoxic molecules leads to killing of the infected target cells. Conversely, antigens derived from extracellular pathogens are taken up by phagocytosis and presented by DCs on MHC class II molecules resulting in activation of $CD4^+$ $T_H$ cells. $T_H$ cells do not only stimulate innate cells such as macrophages but also play a crucial role in activating other adaptive cells, like B cells and $CD8^+$ CTLs thereby contributing to the elimination of the pathogen.

Summing up, innate and adaptive immune responses intensively cooperate with each other thereby both contributing to pathogen clearance. While the innate immune system is crucial during the early phase of a primary infection but does not provide immunological memory, the adaptive immune system takes longer to be activated but endows with immunological memory, which allows an even quicker response in case of re-exposure to the same pathogen.

## 1.2 General aspects of NK cell biology and activation

NK cells are large granular lymphocytes able to kill malignant, infected or ‘stressed’ cells and to produce inflammatory cytokines. Therefore, they play an important role in the elimination of pathogens and tumours. In particular, NK cells contribute to the defence against parasites and intracellular bacteria, and they are critical for controlling several types of viral infections (2, 3). Under steady state conditions, NK cells are widely distributed in lymphoid and non-lymphoid organs, but can rapidly migrate to inflamed tissue in response to different chemoattractans (2).

In contrast to responses of the adaptive immune system, the NK cell response is immediate, and plays its most important role during the first few days of infection with pathogens.

Unlike T and B cells, the specificity of NK cells for target cells is not determined by single antigen receptors encoded by genes undergoing somatic recombination. Instead, NK cells use a range of activating and inhibitory receptors similar to other innate immune cells. The main effector functions of NK cells, such as cytokine production and cytotoxicity, are tightly regulated by a balance between these activating and inhibitory signals. Thus, after engagement of multiple activating receptors expressed on their surface by ligands up-regulated on transformed or infected cells, NK cells can produce cytokines and kill target cells whereas engagement of inhibitory receptors by MHC class I molecules prevent normal cells from killing by NK cells (Figure 1).

Expression of ligands for activating NK cell receptors by pathogen-infected cells or tumour cells can stimulate NK cells, which subsequently kill the target cell by the release of lytic granules. Those granules include perforin and granzymes, which induce the generation of pores in the cellular membrane and the activation of caspase cascades resulting in apoptosis of the target cell (Natural cytotoxicity). The cytotoxic pathway can also be initiated after engagement of the constant fraction γ receptor III (FcγRIII, CD16) expressed on the surface of NK cells by antibodies bound to a cellular target (ADDC) (5), allowing NK cells to specifically kill infected cells with the help of the adaptive immune system.

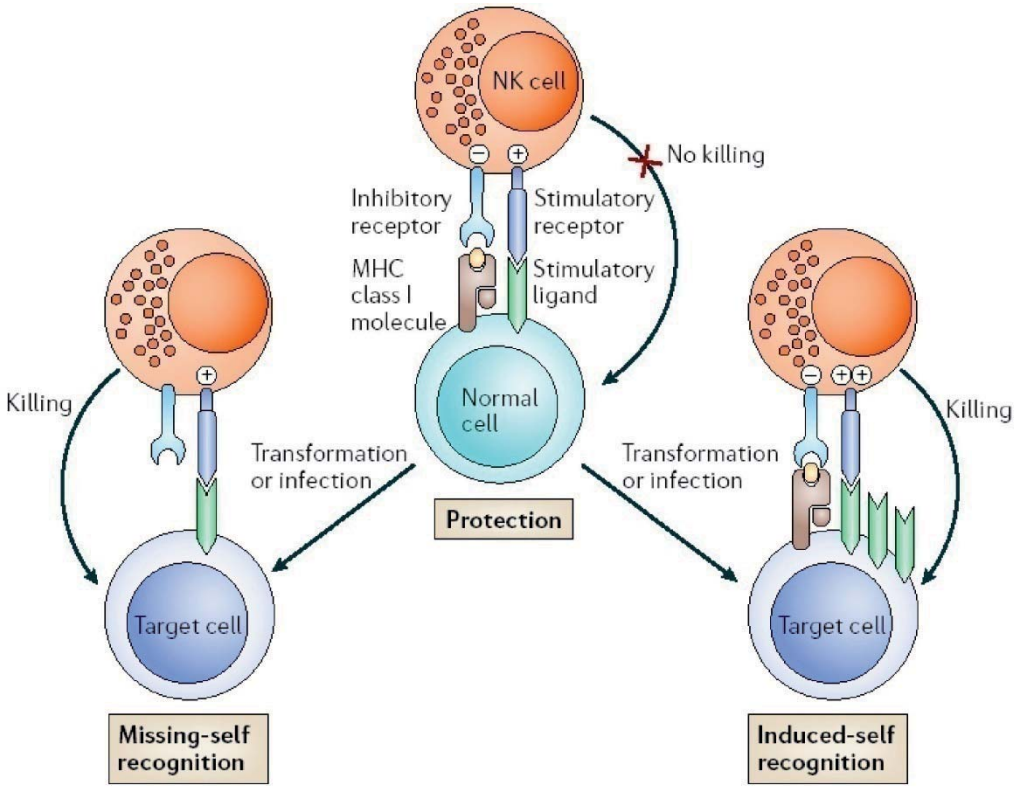


**Figure 1: The balance of activating and inhibitory signals determines NK cell - target cell interaction.**

NK cells are tolerant to cells expressing normal levels of self-MHC class I molecules and low levels of activating ligands. If this balance is distorted by down-regulation of MHC class I, killing is mediated due to missing self-recognition. Conversely, up-regulation of activating ligands leads to killing due to induced self-recognition, even when MHC class I is still present on the cell surface. Figure adapted from Raulet *et al* (4).

In addition, activated NK cells represent an early source of cytokines, especially Interferon-γ (IFN-γ), tumour-necrosis-factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) which lead to the recruitment of other immune cells and to the activation of antiviral responses in infected cells (6). Furthermore, they enhance production of toxic metabolites in macrophages and induce DC maturation and production of Interleukin (IL)-12 in DCs. Through production of cytokines, NK cells are also able to shape the adaptive immune response by driving the differentiation of naive T cells into IFN-γ producing $T_H1$ cells (2).

Although NK cells apparently display the ability to become directly activated by diseased cells, it has been postulated that for proper NK cell priming, APC are required. Thus, NK cells can be activated by DC-derived cytokines such as IL-15 and they can produce IFN-γ in response to IL-12 and IL-18 in the absence of additional activating signals. In addition, IL-2, a cytokine mainly produced by T cells, can activate NK cells resulting in extensive proliferation and maturation.

### 1.3 **NK cells subsets and differentiation**

Human NK cells are defined phenotypically by expression of CD56 and lack of CD3 and comprise approximately 10-15% of lymphocytes circulating in the blood (7). Based upon their cell-surface density of CD56, two populations of human NK cells can be distinguished. The majority (95%) of human peripheral blood (PB)-NK cells express low-levels of CD56 ($CD56^{dim}$), high levels of the FcγRIII CD16 and lytic granules to rapidly mediate cytotoxicity (8, 9). The remaining PB-NK cells (≤ 5%) are represented by $CD56^{bright}$ cells (10), which conversely express high density of CD56 and very low levels of lytic granules, secrete larger amounts of cytokines and proliferate much more vigorously than $CD56^{dim}$ NK cells upon activation (Figure 2).

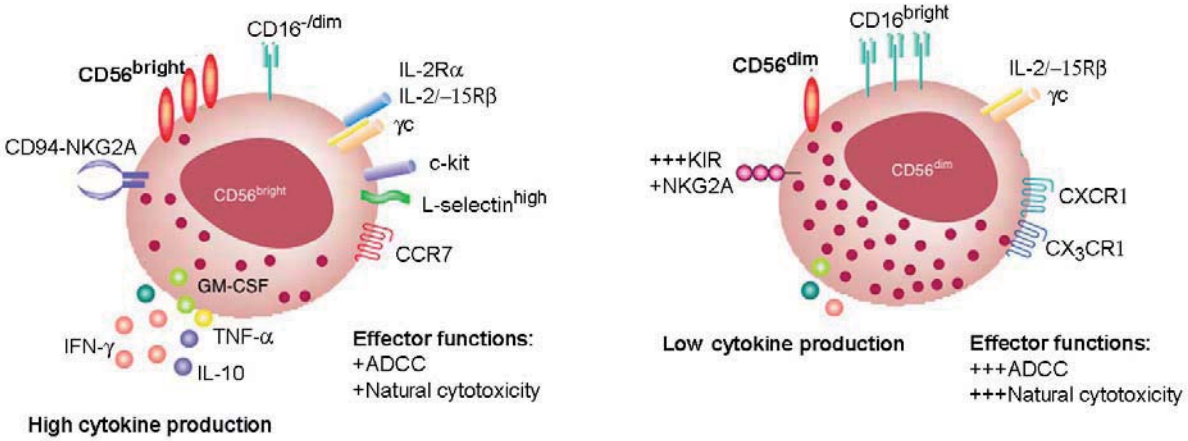


**Figure 2: Schema of human NK cell subsets**

$CD56^{bright}$ cells express low levels of lytic granules and Fcγ receptor III (CD16), no killer cell Ig-like receptor (KIR), but high levels of the inhibitory CD94-NKG2A C-type lectin NK receptor (NKR). $CD56^{bright}$ secrete large amounts of cytokines and proliferate much more vigorously than $CD56^{dim}$ NK cells upon activation with cytokines. In contrast, $CD56^{dim}$ display high levels of CD16, KIR and lytic granules to rapidly mediate cytotoxicity. Figure adapted from Cooper *et al* (10).

### *1.3.1 NK receptors*

NK receptors specific for MHC class I molecules are crucial for distinguishing normal cells from transformed and/ or foreign cells and they exist in both activating and inhibitory forms. In most cases, ligands for activating receptors are pathogen-encoded molecules or self-proteins whose expression is up regulated in transformed or infected cells while ligands for inhibitory receptors are polymorphic variants of MHC class I molecules expressed by all normal cells.

Inhibitory receptors include killer immunoglobulin-like receptors (KIRs) in humans recognizing subclasses of human leukocyte antigen (HLA)-A, -B and -C alleles; Ly49 C-type lectin superfamily receptors in mice and CD94/NKG2 (11) heterodimers specific for HLA-E in both species (12, 13). The engagement of inhibitory receptors leads to tyrosine phosphorylation of immune tyrosine-based inhibitory motifs (ITIM) that recruit tyrosine phosphatases such as SHP-1 (Src homology2 domain-containing phosphatase) and SHP-2, which suppress NK cell responses by dephosphorylating the protein substrates of the tyrosine kinases linked to activating receptors of NK cells (14).

Activating receptors consist of Ig superfamily members such as activating KIRs, CD16, CD2, 2B4 and the natural cytotoxicity receptors (NCR) NKp46, NKp30 and NKp44 and of member of the C-type lectin superfamily such as NKG2D. CD16, also known as FcγRIII, represents a low affinity receptor for the Fc portion of antibodies belonging to the IgG isotype (15) and mediates antibody dependent cytotoxicity (ADCC), whereas natural cytotoxicity is mediated by NCRs, NKG2D, CD2 or 2B4 (16). Remarkably, human NK-cell subsets differ in their expression of NKRs (17, 18), suggesting unique regulation of cytotoxic properties. Thus, $CD56^{bright}$ NK cells do not express KIRs and have high-levels of CD94-NKG2A expression, whereas the opposite is the case for $CD56^{dim}$ NK cells (17, 18). Also CD16 is present on most of the $CD56^{dim}$ NK cells while 50-70% of $CD56^{bright}$ are negative for CD16 or express it at low density (10). In contrast, the activating receptor NKG2D and activating NCRs are equally expressed on both NK-cell subsets (19, 20).

### *1.3.2 Cytokine and chemokine receptors and adhesion molecules*

All NK cells express the low-affinity interleukin-2 receptor (IL-2R), comprised by the IL-2Rβ and the IL-2Rγ chain. In addition, $CD56^{bright}$ NK cell are the only lymphocytes that constitutively express the high-affinity heterotrimeric IL-2R (IL-2Rαβγ) (21, 22). Consequently, this subset displays *in vitro* and *in vivo* a high proliferative response to low (picomolar) doses of IL-2 alone (23, 24). Furthermore, $CD56^{bright}$ NK cells, unlike $CD56^{dim}$, express the receptor for stem cell factor (c-kit or CD117) and the alpha chain of the IL-7 receptor, whereas $CD56^{dim}$ do not. Both subsets of NK cells constitutively express several receptors for monocyte-derived cytokines (monokines), including IL-1, IL-10, IL-12, IL-15 and IL-18 (19). For interleukin-18 receptor (IL-18R) and IL-1 receptor (IL-1R), higher expression levels have been shown in the $CD56^{bright}$ subset (25).

NK cells express several chemokine receptors and again $CD56^{bright}$ and $CD56^{dim}$ NK cells display distinct repertoires of these receptors (8). So, $CD56^{bright}$ express secondary lymphoid organ (SLO) homing markers, namely CCR7, L-Selectin (CD62L) and CXCR3 (10, 19, 26), whereas $CD56^{dim}$ lack these receptors and therefore do not have the potential to migrate into SLO. The expression of SLO homing markers together with the constitutive expression of the high-affinity IL-2R highlights the possibility of cross-talk between $CD56^{bright}$ NK cells and T cells in SLO, since IL-2 is produced only by T and not by NK cells (10, 27).

In contrast, $CD56^{dim}$ lack CCR7 and CXCR3 expression and are mainly CD62L negative, but show high levels of CXCR1 and $CX_3CR1$ expression, molecules important for migration to inflamed tissues (8).

### *1.3.3 Functional responses*

NK cells can be activated in two ways. On the one hand, they proliferate and produce cytokines in response to cytokine stimulation. On the other hand, they can be stimulated via engagement of activating receptors, which results in cytotoxicity and cytokine production.

Consistent with their expression of the high affinity IL-2R, the $CD56^{bright}$ NK-cell subset is able to proliferate in response to low doses of IL-2. On the contrary, $CD56^{dim}$ NK cells need much higher IL-2 concentrations for a significant proliferative response. IL-15, which acts via the IL-2/15Rβγ, can also stimulate proliferation of $CD56^{bright}$ and to lower degree of $CD56^{dim}$ NK cells (28).

Due to the constitutive expression of for numerous monokine receptors, NK cells rapidly produce cytokines in response to stimulation by monokines (28-30). $CD56^{bright}$ are considered to be the primary source of NK-cell-derived cytokines, whereas the $CD56^{dim}$ NK-cells are poor cytokine producers following cytokine stimulation *in vitro* (10). This observation suggest that the major function of $CD56^{bright}$ NK cells during the innate immune response *in vivo* might be to provide macrophages and other APCs with early IFN-γ and other cytokines, promoting a positive cytokine feedback loop and efficient control of infection (10).

Activation of NK-cell cytotoxicity is thought to be mediated by a balance of inhibitory and activating signals (Figure 1). As it could be expected by the higher expression levels of lytic granules in $CD56^{dim}$ NK cells, they are naturally more cytotoxic than $CD56^{bright}$ ones (8, 9). However, after activation with IL-2 or IL-12 *in vitro*, or following low dose therapy with IL-2 *in vivo*, $CD56^{bright}$ and $CD56^{dim}$ cells have similar cytotoxic capacity (21, 22, 31). NK cells mediate ADCC also, which requires the activation and engagement of the FcγRIII by Antibody-coated target cells (32). $CD56^{dim}$, expressing high-levels of CD16, exhibit greater levels of ADCC compared to the $CD56^{bright}$ NK cells (33).

## 1.4 Mechanism of NK cell self tolerance

NK cells were originally identified by their ability to spontaneously kill certain tumour target cells *in vivo* and *in vitro* without prior sensitization, if these tumour cells express low levels of MHC class I molecules at their surface. MHC class I proteins can be down regulated in case of infection by viruses or malignancies as a mechanism to escape from the immune system. In this case sensing lack of MHC class I on a target cells results in “missing-self” recognition by NK cells (34).

MHC class I-specific inhibitory receptors provide a molecular mechanism for missing-self recognition. Following inhibitory receptor engagement, NK cell cytotoxicity and cytokine production are inhibited or abrogated. However, inhibitory receptors do not play alone the decisive role in NK cell- target cell interaction. NK cells express activating receptors in addition and upregulation of ligands for these receptors on infected or transformed cells in some cases is sufficient to render a target cell sensitive to NK cell mediated killing, even if it expresses a full set of MHC class I molecules. Thus, the balance between activating and inhibitory signals the NK cell receives from a given target cell decides whether a NK cell becomes activated or not to produce inflammatory cytokines and/or kill the target cells (Figure 1), (12, 13, 35).

In general, each inhibitory receptor is expressed randomly by only a subset of NK cells. As a result, the NK cell population consists of many subpopulations expressing various combinations of the available inhibitory receptors specific for distinct polymorphic variants of MHC class I molecules and consequently have a distinct pattern of reactivity. MHC class I molecules show enormous allelic variations and individual inhibitory NK cell receptors bind certain MHC class I variants and not others. Since the genes for inhibitory receptors and their cognate MHC class I ligands are inherited independently, genetic mechanisms cannot ensure that each NK cell inhibitory receptor encounters its specific self-MHC class I molecule (36). In line with this fact, some NK cells exist which do not express any self-MHC class I specific inhibitory receptor. Because such cells are potentially autoreactive, developmental processes must exist that either repress the activity of such cells or prevent them from developing or maturing in the first place.

Recent studies have shown that such NK cells are hyporesponsive after engagement of activating receptors and therefore self tolerant. Conversely, NK cells, which express inhibitory receptors for self-MHC, are functionally competent (37, 38). Thus, in humans, it has been shown that KIR2DL2/L3$^{+}$, KIR2DL1$^{+}$ or KIR3DL1$^{+}$ CD56$^{dim}$ NK cells derived from donors expressing the corresponding HLA class I ligands (group C1 alleles, group C2 alleles, and HLA allotypes with the Bw4 epitope, respectively) are more responsive to CD16 cross-linking and stimulation with class I negative target cells than other NK cell subsets (39, 40). These data imply that expression of self-specific inhibitory receptors is a crucial event during NK cell education and determines whether a cell will end up in the pool of competent or hyporesponsive NK cells. However, the mechanisms underlying NK cell education, are still controversially debated (Figure 3). On the one hand, it has been proposed that engagement of self-MHC specific inhibitory receptors directly confers functionality to NK cells, a process which is referred to as “licensing” (38, 41). Alternatively, it has been suggested that hyporesponsiveness of NK cells lacking self-specific inhibitory receptors is a consequence of persistent stimulation that is not counterbalanced by inhibitory signals. In this model, potentially autoreactive NK cells would be silenced by the lack of inhibition (“disarming model”) (4, 38, 41, 42).

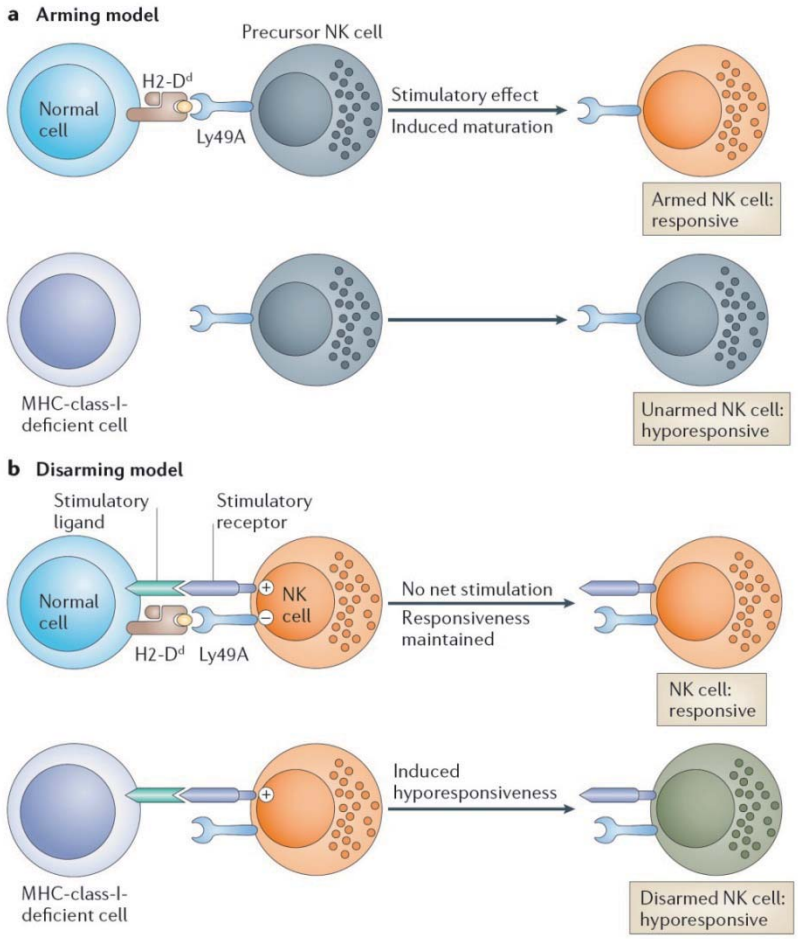


**Figure 3: Mechanisms of NK cell tolerance**

**(a)** In the arming model, positive signals received by a precursor (immature) NK cell through interactions with MHC-class-I-expressing target cells are required to induce functional competence ('arming') of the cell (**b**) In the disarming model, NK cells express stimulatory and inhibitory receptors. NK cells in which inhibitory and stimulatory signals are balanced are allowed to retain (or acquire) responsiveness. NK cells that receive unopposed positive signals (for example, in an MHC-class-I-deficient host) are 'disarmed' and thereby rendered unresponsive. NK cells could be disarmed during their development or in response to chronic positive signaling. Figure adapted from Raulet *et al* (4).

Moreover, it is still unclear whether the hyporesponsive phenotype is stable or whether it is contextual, meaning that it can be reversed in certain contexts (Figure 4). Indeed, during infection with *Listeria monocytogenes*, NK cells that lack inhibitory receptors for self-MHC can produce as much IFN-γ as competent NK cells (37). Interestingly, NK cells derived from β2-microglobulin deficient mice, which are hyporesponsive after certain activating stimuli, are still able to control cytomegalovirus infection *in vivo* (43).

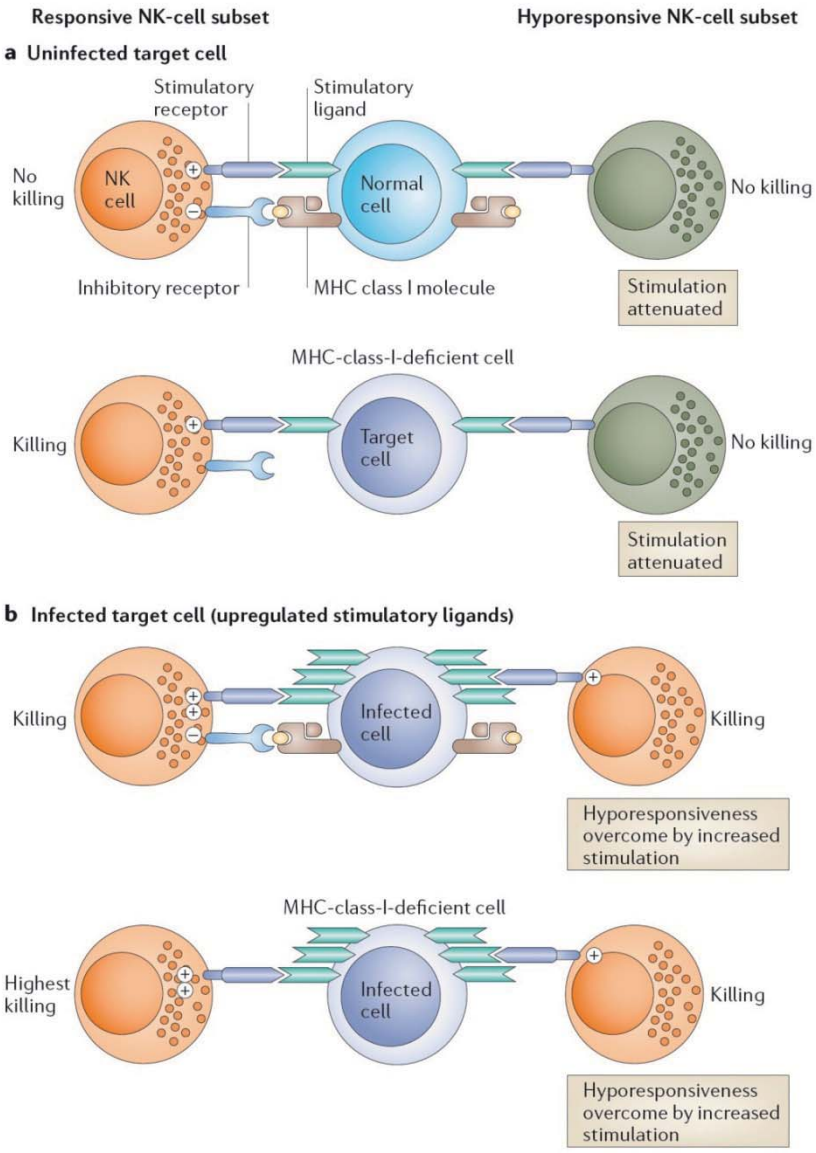


**Figure 4: Contextual hyporesponsiveness**

Depicted are the outcomes of interactions between uninfected target cells **(a)** or infected target cells **(b)** and responsive (left) or hyporesponsive (right) NK-cell subsets. The responsive NK cells express a self-MHC-class-I-specific inhibitory receptor, whereas the hyporesponsive NK cells do not. The hyporesponsive cells do not respond to target cells with decreased expression of MHC class I molecules but can respond to cells with increased expression of stimulatory ligands. Figure adapted from Raulet *et al* (4).

## 1.5 NK subset development

While it is clear that NK cells are part of the hematopoietic system and are derived from $CD34^+$ hematopoietic progenitor cells (HPCs) (44-46), less is known about the sites of development and details of this process. Yet, several studies indicate that both lymph nodes and bone marrow (BM) may be important.

Thus, bone marrow ablation in mice had an adverse effect on NK cell development relative to other hematopoetic lineages. Moreover, IL-15 which has been identified to be the crucial cytokine for NK cell development and maintenance in humans and mice, is expressed by primary bone marrow stromal cells and culture of human bone marrow derived HPCs in IL-2 or IL-15 results in the generation of $CD56^{bright}$ NK cells (47). Two additional BM stromal factors, ligands for the receptor tyrosine kinases c-kit and flt-3, have also been shown to act synergistically with IL-15 to enhance NK cell expansion in culture (48).

Nonetheless, NK development might not occur completely in the bone marrow, since $CD56^{bright}$ NK cells are enriched in all secondary lymphoid organs compared to $CD56^{dim}$ which are mainly found in bone marrow and blood (49, 50). Like PB-$CD56^{bright}$ NK cells, SLO-NK cells exhibit no KIR or CD16 expression and poor cytolytic activity. Therefore, it has been postulated that this SLO-$CD56^{bright}$ NK population was less mature than the PB-$CD56^{dim}$ subset (51). Furthermore, $CD34^+$ $CD45RA^+$ pre-NK cells present at low frequencies in BM and in blood were found to be enriched highly and selectively in SLO (52). This enrichment of both pre-NK cells and $CD56^{bright}$ NK cells within SLO relative to BM or blood, together with the presence of DCs capable to trans-present IL-15, suggest that SLO may be another site for NK-cell development *in vivo* (53). Indeed, phenotypically and functionally distinct cell populations that represent stages along the NK- cell developmental pathway from $CD34^+$ $CD45RA^+$ HPCs to $CD3^-CD56^{bright}$ NK cells were discovered *in situ* (54, 55).

With its higher intrinsic cytotoxicity, more abundant expression of CD16 and lower proliferative response, $CD56^{dim}$ NK cells appears to be more terminally differentiated than $CD56^{bright}$ NK cells. The facts that in vivo therapy with IL-2 generates a predominance of $CD56^{bright}$ NK cells (24) and that this subset is the first one reoccurring after bone marrow transplantation (56) promote the hypothesis that $CD56^{dim}$ cells derive from $CD56^{bright}$ ones in the periphery (10).

In this regard, controversial hypothesis have been proposed for the developmental relationship between these two NK cell subset, since CD56$^{bright}$ NK cells have been suggested to represent either precursors of CD56$^{dim}$ cells or to be derived from CD56$^{dim}$ cells (Figure 5) (51, 55, 57-59). Studies addressing this question have been hampered by the lack of CD56 in mice, by missing informations regarding the site of terminal NK cell differentiation and by the lack of markers, which together with CD56 expression would identify NK cell subsets with intermediate phenotype and functional properties.

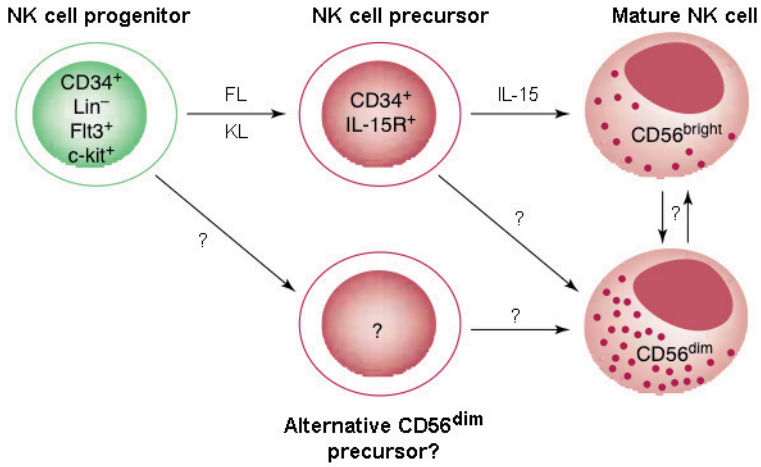


**Figure 5: Human NK-cell subset development**

NK-cell development can be divided into three discrete stages based on *in vitro* models: CD34$^{+}$ NK-cell progenitor, responsive to flt3 ligand (FL) and/or c-kit ligand (KL) differentiates into a CD34$^{+}$ interleukin-15 receptor (IL-15R)$^{+}$ NK precursor which then differentiates into a functionally mature CD56$^{bright}$ NK cell. The developmental relationship between CD56$^{bright}$ and CD56$^{dim}$ NK cells has never been established definitively, and CD56$^{dim}$ NK cells have not been generated in vitro. Either CD56$^{dim}$ NK cells could develop from a unique CD56$^{dim}$ NK-cell precursor or an alternate signal (e.g. a novel cytokine) could induce the differentiation of CD56$^{dim}$ cells from a common NK-cell precursor or CD56$^{bright}$ cells could maturate into CD56$^{dim}$ NK cells. Figure adapted from Cooper *et al* (10).

### *1.5.1 Heterogeneity of CD56$^{dim}$ NK cells*

Besides the division of labour between CD56$^{bright}$ and CD56$^{dim}$ NK cells, in the last years it became clear that CD56$^{dim}$ NK cells themselves do not represent a homogenous population of effectors ready to proliferate, to produce cytokines or to kill. In contrast, NK cells undergo a differentiation program which includes MHC-dependent education (37-39), priming (60-62) and even generation of memory during recall responses (63, 64). This complexity implies the existence of intermediate stages of NK cell differentiation, which can guarantee an efficient division of labour as it has been shown for cells of the adaptive immune system. Interestingly, CD56$^{dim}$ NK cells are heterogeneous concerning the expression of several markers, namely KIR, NKG2A, CD27 and CD62L. It has been shown that among CD56$^{dim}$ cells, competent or hyporesponsive NK cells can be identified according to the expression of MHC specific inhibitory receptors such as KIR or NKG2A. On the other hand, it is reported that also CD27 expression influences NK cell functions as it correlates with high ability to proliferate and to produce IFN-γ and with low cytotoxic potential, at least in humans (65, 66). However, functional analysis considering all these molecules together including CD62L has never been performed so far.

# 2 Aims of the Thesis

Human NK cells can be divided into $CD56^{bright}$ $CD16^{-}$ $KIR^{-}$ $CD62L^{+}$ and $CD56^{dim}$ $CD16^{+}$ $KIR^{+/-}$ $CD62L^{+/-}$ subsets that differ in function, phenotype and tissue localization. While $CD56^{bright}$ NK cells are characterized by the ability to proliferate extensively and to produce IFN-γ in response to cytokines, $CD56^{dim}$ NK cells have high cytotoxic capacity. Nonetheless, the developmental relationship between these two NK cell subsets remains controversial. $CD56^{bright}$ NK cells have been suggested either to be precursors of $CD56^{dim}$ cells or to derive from $CD56^{dim}$ cells.

Interestingly, $CD56^{dim}$ NK cells themselves do not represent a homogeneous population concerning the expression of several surface molecules, such as inhibitory receptors, CD62L or CD27 and concerning functional properties. Thus, the expression of at least one self-MHC specific inhibitory receptor correlates with higher ability to kill and to produce IFN-γ in response to stimulation via activating receptors. Engagement of inhibitory receptors by cognate MHC molecules is the mechanism by which NK cells mediate tolerance towards self and acquisition of inhibitory receptors by NK cells is a critical prerequisite for the generation of functional competent NK cells. Whether or not hyporesponsiveness of NK cells can be overcome under certain circumstances is not known so far. In addition to inhibitory receptors other molecules, such as CD27, which has been correlated with high ability to proliferate and to produce IFN-γ, or CD62L, a lymph node homing marker, are differentially expressed within $CD56^{dim}$ NK cells. Both of them are commonly used to identify distinct T cell maturation stages

The aim of this thesis was to investigate whether PB-$CD56^{bright}$ NK cells can differentiate *in vitro* and *in vivo* to cells analogous of $CD56^{dim}$ NK cells and whether SLO can be sites of NK cell maturation. Moreover, it was analysed whether cytokines can induce expression of self specific inhibitory receptors and if so whether that would be sufficient to educate hyporesponsive NK cells to become competent and self tolerant. The aim of the third part of my thesis was to evaluate whether the expression of CD62L or other markers might be used to identify intermediate stages of NK cell maturation characterized by distinct functional properties and in line with that better define NK cell developmental history.

# 3 Materials and Methods

## 3.1 Buffers, Medias and solutions

Table 1: Buffers, Media and solutions

| Buffer | Description and company |
|---|---|
| PBS-buffer<br>(Phosphat-buffered Saline) | 1,5 mM $KH_2PO_4$<br>2,7 mM KCL<br>8,1 mM $Na_2HPO_4$<br>137 mM NaCL<br>pH: 7,2- 7,4 |
| PBS/BSA-buffer: | 5g/l (0,5%) Bovine serum albumin (BSA Boehringer-Mannheim, Germany) in PBS-buffer |
| PBS/BSA/EDTA | 2 mM ethylene diaminetetraacetic acid (EDTA) in PBS/BSA buffer |
| RMPI medium: | Rosewell Park Memorial Institute Medium (RPMI) 1640 (Gibco BRL, USA), supplemented with 100 U/mL penicillin and 0.1 mg/ml streptomycin and either<br>10% human AB serum (Lonza, USA) or<br>10% foetal calf serum (FCD, Sigma Aldrich, Germany) |
| Ficoll: | Lymphocyte-Separations-Medium (Ficoll-Paque™ PLUS Amersham Pharmacia Biotech AB, Sweden) |
| FACS-Perm2: | FACS™ -Perm-Solution (BD), diluted 1:10 in Aqua dest |

## 3.2 Devices and materials

Table 2: Devices and material

| **Device or material** | **Description and company** |
|---|---|
| FACS-tubes | Falcon 5ml, round bottom (BD Bioscience) |
| Reaction tubes | Safe-Lock-Tubes 0,5ml; 1,5ml; 2ml<br>(Eppendorf) |
| Cell filter | CellTrics 30 µm, (Partec, Germany) |
| Lamina flow box | HERA safe (Heraeus, Germany) |
| Flow Cytometer | FACS LSRII (BD Bioscience) |
| FACS (Cell-Sorter) | FACSAria (BD)<br>FACSDiva (BD) |
| MACS (Cell-Sorter) | AutoMACS (Miltenyi Biotec) |
| Centrifuges | Multifuge 3 S-R (Heraeus, Germany)<br>Biofuge fresco (Heraeus, Germany<br>Megafuge 1.0 (Heraeus, Germany) |
| Cell Counter | Casy DT (Schärfe Systems, Germany) |
| Incubator | Cell culture incubator (Heraeus, Deutschland) |
| 96-er Microtiterplates (round bottom) | Greiner Bio-one PS-Microplate 96K (ELISA) |
| Software | FlowJo (Tree Star Inc.) |

## 3.3 Chemicals

Table 3: Chemicals

| **Chemical** | **Company** |
|---|---|
| Brefeldin A (Bref-A)<br>(5mg/ml in 70% ethanol) | Sigma-Aldrich, Germany |
| Carboxyfluoresceindiacetat (CFDA)<br>5mM in Dimethylsulfoxid (DMSO) | Molecular Probes, Netherlands |
| Monensin | BD Bioscience, Germany |
| EDTA<br>(2mM in PBS-Buffer) | Merck, Germany |
| 4,6-Diamidin-2-Phenylindol-Dihydrochlorid (DAPI)<br>(1µg/ml in PBS-Buffer) | Roche, Germany |
| Streptavidine (SA)-Pacific Blue<br>SA-Alexa 647<br>SA-PE | Molecular Probes |

## 3.4 Cell culture conditions

All experimental procedures were performed under sterile conditions in a Laminar-Flow-Box (HERA safe, Heraeus, Germany).

$5x10^5$/ml primary human cells were cultured in RPMI medium with 10% AB serum in 96-well round bottom plates (Greiner bio-one, Germany) at 37°C and 5% $CO_2$ in a cell culture incubator (Heraeus, Germany). Tumour cell lines were cultured in RPMI medium with 10% FCS in 10 ml cell culture flasks (Cellstar, Greiner bio-one, Germany) under the same conditions.

Cell counts were calculated with a Casy DT cell counter (Schärfe Systems, Germany) according to manufacturer's instructions.

## 3.5 Cell isolation and cytometric analysis

### *3.5.1 Isolation of Peripheral blood mononuclear cells (PBMC)*

Whole blood contains erythrocytes, granulocytes, PBMC, which comprise lymphocytes (B cells, T cells and NK cells), monocytes and dendritic cells. PBMC can be separated from erythrocytes, granulocytes and plasma by density gradient centrifugation with Ficoll, a neutral, highly branched, hydrophilic polysaccharide (p=1.07 g/l). Ficoll has lower density than erythrocytes and dead cells, the same as granulocytes and higher ones than mononuclear cells. After density gradient centrifugation, typical layers formed from top to bottom are plasma, PBMC, Ficoll and granulocytes, and erythrocytes, which are present in pellet form.

For PBMC isolation, Buffy Coats were obtained from healthy donors after donor informed consent and approval by the local ethics committees on human studies (Charité, Berlin, Germany). Blood samples were diluted 1:1 in PBS/BSA and piled up carefully on three 50 ml tubes (BD, Germany) containing 12.5 ml Ficoll-Paque. The samples were centrifuged at 800x g for 20 min at room temperature in a Multifuge centrifuge (Heraeus, Germany) without brake. The upper plasma layer was removed until 1 ml above the typical white layer containing the PBMC. PBMC were transferred into new 50 ml tubes; these were filled up with PBS/BSA and centrifuged at 300x g for 10 min at 4°C. The supernatant was discarded and the second washing step was performed at 175x g for 15 min in order to remove thrombocytes. After discarding the supernatant, cells were used for further experiments.

### *3.5.2 Magnetic activated cell sorting (MACS®)*

For enrichment and isolation of cell populations, Magnetic cell separation was employed. The MACS® technology (Miltenyi Biotec GmbH, Germany) is based on the labelling of cell surface molecules with antibodies coupled to superparamagnetic particles of approximately 50nm in diameter („Microbeads“) and uses MACS® Columns for cell separation. These columns are placed in a strong permanent magnet (MACS® Separator) and a high-gradient magnetic field is induced on the column matrix. The cell suspension containing labelled and unlabelled cells is placed on the column, and while unlabelled cells pass through and can be collected as negative fraction, labelled cells are bound to the matrix and are released after removal of the column from the magnet as positive fraction. Thus, MACS® Technology can be used for enrichment or isolation (positive selection) or depletion (negative selection) of cells. The cells of interest can be labelled either directly with antibodies coupled to magnetic beads or indirectly by labelling the cells first with a primary antibody and subsequently with a secondary antibody directed against the isotype of the primary antibody or the fluorochrome, which is coupled to the primary antibody.

For pre-enrichment of human total NK cells, $1x10^9$ PBMCs were incubated with CD56 MicroBeads (Miltenyi Biotech, Germany) and subsequent positive selected using the Automacs Separator (Miltenyi Biotech, Germany). For some experiments, NK cells were enriched by negative selection using NK cell isolation Kit II from Miltenyi, a so called “untouched sort”, where all cells apart from NK cells are magnetically labelled and depleted.

For isolation of circulating myeloid dendritic cells (mDCs), PBMCs were first stained with anti-CD1c-FITC antibodies and were subsequently sorted using anti-Fitc MicroBeads (Miltenyi Biotech, Germany) (Staining protocol see 2.4.3).

$CD4^+$ T cells were enriched after staining of PBMCs with CD4 Microbeads followed by positive selection with the Automacs.

Briefly, $1x10^9$cells/ml were incubated for 15min at 4°C with the respective beads added at a final dilution of 1:5 in PBS/BSA/EDTA. All washing steps were performed at 300 x g and 4°C for 10min using PBS/BSA/EDTA. Before MACS, cells were pre-separated (30µm filter, Partec, Germany) in order to protect MACS columns from plugging. Cells were separated using the Automacs program possel_s (positive selection in sensitive mode), a program applied for the enrichment of rare cell populations. This program facilitates high recovery of cells, although with lower purity. In case of the negative selection, NK cells are found in the flow through. All reagents used for MACS are depicted in Table 4.

Table 4: MACS reagents

| | |
|---|---|
| α-CD56 Microbeads | Miltenyi Biotec |
| α-CD1c Fitc | Miltenyi Biotec |
| α-Fitc Microbeads | Miltenyi Biotec |
| NK cell isolation Kit II | Miltenyi Biotec |
| α-CD4 Microbeads | Miltenyi Biotec |

### *3.5.3 Fluorescence Activated Cell Sorting (FACS) and flow cytometric analysis*

Flow cytometry developed from fluorescent microscopy where cell surface molecules are labelled with monoclonal antibodies coupled to fluorescent dyes. With the help of this method, surface and intracellular molecules can be detected. Immunofluorescence staining can be employed either to analyse cells using flow cytometry or to sort cell populations with FACS.

The principle underlying single cell analysis is hydrodynamic focussing of the cells in suspension. One cell at a time is passing a capillary and the fluorochome coupled to the antibody used for staining becomes excited by a laser beam. At present four different lasers, blue-, red-, violet and UV-laser, are used in one analyser. In order to determine the relative size of the cells, forward scattered light (FSC) is detected from the opposite site of the illuminating light. In an angle of 90°C side scatter (SSC) and the emission wavelengths of the excited fluorescent dyes are detected. The side scatter provides information on the granularity of cells.

Once a fluorochrome becomes excited with the defined wavelength of the laser, the emitted light is collected and passes long pass filters, which reflect all the wavelengths shorter than the indicated one. Afterwards the transmitted light passes band pass filters that allow transmission of a certain area of wavelengths and thus fine-tune the spectral wavelengths that are finally detected. In the end, the signal hits the photomultiplier, becomes amplified 1000 to 1 million folds depending on the supplied voltage, is converted into electrical current pulses and detected (Radbruch, Flow cytometry and cell sorting 2$^{nd}$ Ed.).

Cells cannot only be analysed, but they can as well be sorted according to differential marker expression using high voltage deflection plates, which deflect side streams during sorting.

Although many different fluorescent dyes with distinct excitation and emission spectra are available, the emission spectra of some fluorochromes partly overlap, leading to the detection of one dye in more than one detector. In order to overcome this inconvenience, compensation is required.

The configuration of long pass and band pass filters and the corresponding fluorescent dyes used in this study are summarized in Table 5.

Table 5: Fluorochromes and filter sets

| **Fluorochrom** | **Laser** | **Longpass-Filter** | **Bandpassfilter** |
|---|---|---|---|
| FITC | 488-nm blue Laser | 505 | 530/30 |
| PE | 488-nm blue Laser | 550 | 575/26 |
| PerCp | 488-nm blue Laser | 635 | 670/14 |
| PE-Cy7 | 488-nm blue Laser | 735 | 780/60 |
| Pacific Blue;<br>DAPI | 405-nm violet Laser | - | 440/40 |
| APC<br>Cy5<br>Alexa 647 | 633-nm red Laser | - | 660/20 |
| APC-Cy7 | 633-nm red Laser | 680 | 680/30 |

In order to obtain high purity sorting of different cell subsets, MACS enriched cell fractions were stained with mouse-anti-human monoclonal antibodies either coupled to fluorescent dyes or to biotin, which in a second step can be labelled with a streptavidin-dye conjugate.

In Table 6, all antibodies used in the experiments are listed. “2D KIR” includes KIR2DL1/S1/L2/S2/L3, “3D KIR” includes KIR3DL1/S1/L2 and “2+3DKIR” the combination of 2D KIR and 3D KIR.

Table 6: Antibodies

| **Antibody (mouse-anti-human)** | **Label** | **Clone** | **Company** |
|---|---|---|---|
| α-CD56 | PE-Cy7<br>PE<br>APC | NCAM16.2 | BD Bioscience<br>Miltenyi Biotec<br>Miltenyi Biotec |
| α-CD3 | Fitc<br>Cy5 | UCHT-1 | DRFZ |
| α-CD3 | PerCP | UCHT-1 | Biolegend |
| α-KIR3DL1 | PE<br>PE | DX9 | Biolegend<br>Miltenyi Biotec |
| α-KIR2DL1/S1 | Cy5<br>biotin | EB6 | DRFZ |
| α-KIR2DL2/L3/S2 | Cy5<br>biotin | GL183 | DRFZ |
| α-KIR3DL1/ L2/S1 | Cy5<br>biotin | AZZ158 | DRFZ |
| α-CD62L<br>α-CD62L | PE<br>Fitc<br>APC | Dreg 56<br>Dreg 56 | BD Bioscience<br>BD Bioscience |
| α-NKG2A | Cy5<br>biotin | Z270 | DRFZ |

| | | | |
|---|---|---|---|
| α-CD107a | Fitc | 1D4B | BD Bioscience |
| α-BW4 | Fitc | 0.L.6 | USBiological |
| α-CD19 | APC | 1D3 | BD |
| α-CD14 | Cy5 | TM1 | DRFZ |
| α-CD4 | PerCp | TT1 | DRFZ |
| α CD27 | PE<br>Cy5 | 2E4 | DRFZ |
| α CD69 | Fitc | L78 | BD Bioscience |
| α-CD127 | PE | R34.34 | Beckman Coulter |
| α-NKp46 | PE | 9E2 | Miltenyi Biotec |
| α-NKp30 | APC | 2.29 | Miltenyi Biotec |
| α-NKG2D | APC | BAT221 | Miltenyi Biotec |
| α-CCR7 | PE<br>Fitc | 150503 | R&D System |
| α-IgG2a | biotin | | Southern Biotech |
| α-CD45RA | PE-Cy7 | L48 | BD Pharmingen |
| α-CD45RO | Fitc | UCHL1 | BD Pharmingen |
| α-CXCR3 | APC | 1C6 | BD Pharmingen |
| α-CD117 | APC | YB5.B8 | BD Pharmingen |
| α-CD16 | PE<br>APC-Cy7 | 3G8 | BD Pharmingen |
| α-granzymeA | PE | CB9 | BD Pharmingen |

| | | | |
|---|---|---|---|
| α-granzymeB | Alexa Fluor 647 | GB11 | BD Pharmingen |
| α-granzymeB | PE | GB12 | Caltag |
| α-perforin | APC | dG9 | Biolegend |
| α-Ki67 | Fitc | 35/Ki67 | BD Bioscience |
| α-IFNγ | APC<br>Fitc | B27<br>4SB3 | BD Pharmingen<br>DRFZ |
| α–TNFα | FITC | MabII | BD Pharmingen |
| α-IL-15Rα | Biotin | JM7A4 | Biolegend |
| α-CD122 | PE | Mik-β3 | BD Pharmingen |
| α-CD132 | Biotin | TUGm2 | BD Pharmingen |
| α-STAT5 (pY694) | Alexa-Fluor 647 | 47 | BD Pharmingen |
| α–CXCR1 | APC | | BD Pharmingen |

Up to $1x10^7$cells/100µl were stained in the dark for 10min on ice (in case of intracellular staining for 20min at room temperature) and afterwards washed with PBS/BSA. Propidium iodide (PI) or DAPI, which intercalates the DNA of dead cells, was added to the cells before sorting or analyzing them in order to exclude non-viable cells (not when cells were fixed for intracellular staining). Cells were sorted using a FACSAria cell sorter (BD Biosciences, USA) or analysed at the LSRII$^{TM}$ flow cytometer employing FACSDiva Software (BD Bioscience). Data were analysed using FlowJo software (Tree Star, Inc).

In order to obtain highly purified NK cell subsets, MACS-enriched $CD56^+$ cells from PB or Lymph nodes (LN) were FACS-sorted according to the lack of CD3 and the expression of CD56 and additional markers, when indicated in the text. Histological evaluation of LN was performed by Guido Ferlazzo (University of Messina, Italy).

Briefly, LN were incised immediately after removal and cut into two parts, one of which was paraffin embedded in order to perform histology, while the other was processed for single cell isolation. For isolation of LN single cells, LN were mechanically dissociated and then treated with enzymes, as previously described (50).

For FACS sorting of highly purified mDC, MACS enriched CD1c-Fitc$^{+}$ fraction was further stained with anti-CD19-APC and anti-CD14-Cy5 in order to exclude contamination of B cells or monocytes.

Naive and memory T cells were isolated after positive selection of CD4 cells by MACS followed by FACS sort after staining with anti-CD4, anti-CD45RA, anti-CD45RO and anti-CD27mAbs.

Purity of all sorted subsets was always above 98%.

## 3.6 NK cell stimulation

### *3.6.1 Proliferation assay*

CFDA labelling: up to 1x10$^{7}$ cells/ml were incubated with 0.5μM CFDA in PBS for 4 minutes at room temperature in the dark. Reaction was stopped by filling up the tube with RPMI medium plus 10% AB serum and cells were washed twice.

For analysis of proliferative capacity CFSE labelled NK cells were stimulated for 5 days in the presence of 50ng/ml IL-2 or 50ng/ml IL-15 (R&D) or 50ng/ml IL-12 (Miltenyi Biotec) or together with 2x10$^{4}$ PB-derived mDCs plus 10μg/ml R848 (Alexis Biochemicals) and 100ng/ml LPS (E.coli R515, Ligands set I, Apotech).

### *3.6.2 Analysis of cytokine production*

To analyse IFN-γ production, NK cells were stimulated in the presence of 50ng/ml IL-12 and 50ng/ml IL-18 (Miltenyi biotec) for 28h. 10µg/ml BrefeldinA (Sigma-Aldrich) was added for the last 8 hours. Afterwards cells were fixed by incubation for 10 minutes in a 1,5% formaldehyde (Merck) solution in PBS at room temperature. Following washing with PBS cells were permeabilised with FACSPerm Solution (BD) according to the manufactures' instruction and stained with anti-IFN-γ mAbs for 20min at room temperature. IFN-γ expression was analysed at the LSRII. In some experiments, additional surface markers were stained before fixation and permeabilisation.

### *3.6.3 Stimulation via activating receptors*

To analyse IFN-γ and TNF expression after activating receptor stimulation, goat $F(ab)_2$ anti-mouse IgG (Beckman Coulter) was coated to plastic wells (96 well, PS, U-Bottom, greiner bio-one) for 2 hr in PBS at 37°C at 20 µg/ml. After washes, mouse anti-human NKp30 (AZ20), NKp46 (BAB281), NKG2D (BAT221), 2B4 (PP35) and CD2 (PPA-2.10) specific mAb (kindly provided by S. Parolini, University of Brescia, Italy), were incubated for 30 min at 4°C at 10 µg/ml in PBS. After washes, $2x10^5$ cells/well were plated in each well and stimulated for 8h in the presence of BrefeldinA.

## 3.7 **Analysis of cytotoxic potential**

NK cell cytotoxicity was analysed by cocultivating the indicated NK cell subset with the MHC-class I negative target cell line K562 (ATTC) or when indicated with 721.221, or 721.221 transfectans expressing HLA-B51 or HLA-B7, in an effector to target ratio of 5:1 for 6 h at 37°C.

To measure degranulation as marker for cytotoxic potential the CD107a Mobilization assay was performed, as previously described (67). Briefly, NK cells were stimulated with the target cell line as described above in the presence of anti-CD107a FITC mAb (BD Pharmingen), 0.7µl/ml Monensin (BD Bioscience) and 10µg/ml BrefeldinA. Cells were subsequently stained for surface markers and analysed by flow cytometry.

In some experiments cytotoxicity was directly assessed using a flow cytometric assay for NK cell killing developed by McGinnes et al with slight modifications (68). Briefly, K562 cells were loaded with 5 μM CFDA for 4 min, washed twice and incubated with NK cells. After 6 h each sample was resuspended in a final volume of 250 μl of PBS, to which propidium iode was added. Live target cells were identified by strong green fluorescence whereas dead target sells (Td) showed slightly reduced green and red fluorescence. Specific lysis was calculated as:

$Td_{\text{(cultured with effector cells)}} - Td_{\text{(cultured without effector cells)}}$.

### 3.8 **Analysis of telomere length and telomerase activity**

The indicated NK cell or T cell subsets were sorted from PB as described in 2.4.3, stored in 90% FCS/ 10% DMSO at -80°C and send for analysis of telomere length to Guido Ferlazzo (University of Messina, Italy).

LNs were obtained from cancer patients undergoing surgical resection.

Analysis of telomere length was performed using a quantitative fluorescence in situ hybridization methodology (Flow-FISH) that employs a fluorescein-conjugated peptide nucleic acid (PNA) probe (Telomere PNA Kit/FITC for Flow Cytometry, DakoCytomation, Denmark), according to manufacturer instructions. Briefly, a single cell suspension of sorted NK cells was mixed with control cells (i. e. the 1301 cell line) which display very long telomeres. Mixed cell suspension DNA was denatured for 10 minutes at 82° C either in hybridization solution without probe or in hybridization solution containing fluorescein-conjugated PNA telomere probe. Hybridization took place in the dark at room temperature overnight and was followed by two washes at 40°C. After propidium iodide staining, flow cytometric analysis was performed gating on G0/1-cells. The relative telomere length (RTL) value was calculated as the ratio between the telomere signal of each sample and the control cells (1301 cell line) with correction for the DNA index of G0/1 cells. This correction was performed in order to standardize the number of telomere ends per cell and thereby telomere length per chromosome.

### 3.9 RT-PCR analysis of KIR transcripts

Total RNA was extracted from $CD56^{bright}$, $CD56^{dim}$ $KIR^{-}$ and $CD56^{dim}$ $KIR^{+}$ NK cells at day 0 directly after sorting or after 5 days of culture in the presence of IL-2 using RNeasy micro kit (Qiagen) according to manufacturer's instruction. RNA was send to Michela Falco (Institutio Giannina Gaslini, Genoa, Italy) for RT-PCR analysis of KIR transcripts.

cDNA synthesis was performed on about 500 ng of RNA using oligo (dT) primers. Three different sets of primers were used in this study. KIR up: CAT GTY GCT CAY KGT CGT C and KIR down: GGT TTT GAG ACA GGG CTG allowed the amplification of the KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL2, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1 ORF. The sets of primers common up/C and common up/E allowed the amplification of a segment of activating and inhibitory KIR transcripts respectively, as previously described (69). The PCR products were resolved into 0.8% agarose gel.

### 3.10 Blood samples and Ethical applications

Whole blood and leukocyte concentrates were collected from healthy donors. LN, thoracic duct lymph and blood were obtained from patients who underwent surgery for cancer resection. All sample collections were obtained after donor informed consent and approval by our Institutional Ethics Committee.

For the vaccination study, 8 healthy donors aged 22-53 years without previous yellow fever vaccination or known exposure have been immunized once with YFV 17D (Sanofi Pasteur). Immediately before (day 0) and at day 7 and day 28 after vaccination 20 ml of blood has been drawn and analysed.

### 3.11 **Statistical analysis**

If variables were not Gaussian distributed, Wilcoxon signed rank test was used for statistical analysis. For Gaussian distributed variables, paired student's t test was used for statistical analysis. Gaussian distribution was tested by Shapiro Wilk normality test and D'Agostino & Pearson omnibus normality test. Linear correlation was analysed using the Pearson correlation coefficient. The test used for the individual experiments are indicated in the figure legends.

# 4 Results

## 4.1 CD56$^{bright}$ NK cells display longer telomeres and acquire features of CD56$^{dim}$ NK cells upon cytokine activation

The developmental relationship between CD56$^{bright}$ and CD56$^{dim}$ NK cells is still controversially discussed in the literature. The aim of the first part of this thesis was to investigate whether peripheral blood (PB)- CD56$^{bright}$ NK cells are precursors of and therefore give rise to cells akin of CD56$^{dim}$ NK cells and whether secondary lymphoid organs can be sites of NK cell maturation.

### *4.1.1 Phenotype of peripheral blood NK cells*

Many molecules have been described to be expressed differentially in PB-CD56$^{bright}$ and CD56$^{dim}$ NK cells. As shown in Figure 1 and described in several reports, the majority of PB-NK cells (≥ 95%) belong to the CD56$^{dim}$ CD16$^{+}$ NK subset and express lytic granules such as granzyme A (granzA), granzyme B (granzB) and CD16, the Fcγ receptor IIIA. The remaining PB-NK cells (≤ 5%) are represented by CD56$^{bright}$ CD16$^{-}$ cells (10), which conversely express very low levels of lytic granules and, unlike CD56$^{dim}$, the receptor for stem cell factor (c-kit or CD117), the alpha chain of the IL-7 receptor and secondary lymphoid organ (SLO) homing markers, namely CCR7, CD62L and CXCR3. Notably, the MHC class I allele-specific Killer Ig-like receptors (KIR) are expressed on a considerable fraction of CD56$^{dim}$ CD16$^{+}$ NK cells, while the CD56$^{bright}$ CD16$^{-}$ NK subset lacks KIR.

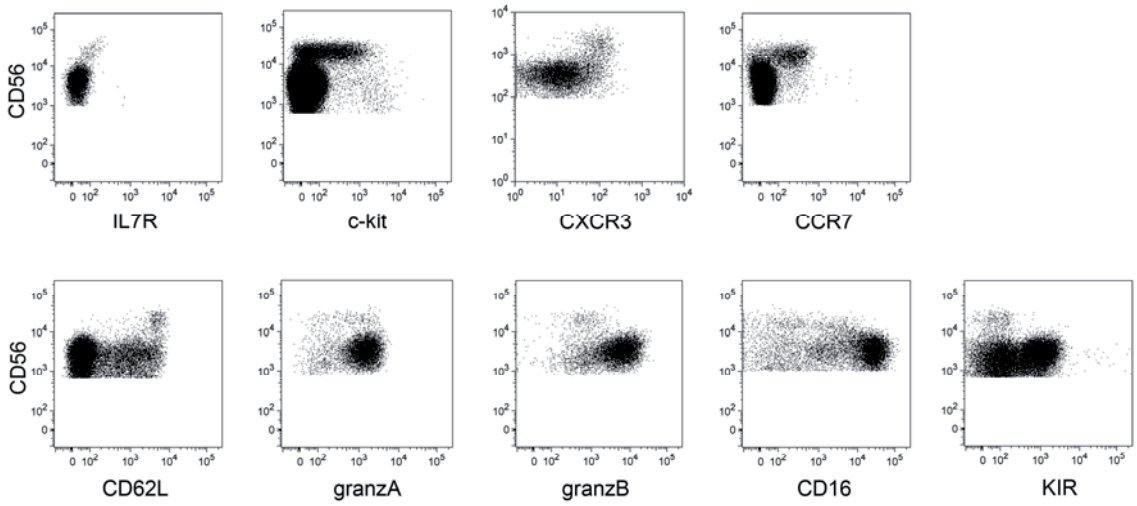


**Figure 1: Phenotype of PB- NK cell subsets**

PBMCs were stained for CD3 and CD56 to gate on NK cells, and in addition for IL7Rα, c-kit, CXCR3, CCR7, CD62L, granzyme A (granzA), granzyme B (granzB), CD16 or 2+3D KIRs (KIR) and analysed by flow cytometry. Expression of these molecules in relation to CD56 density on $CD3^-$ $CD56^+$ NK cells is shown.

### *4.1.2 $CD56^{bright}$ NK cells derived from Peripheral blood and Lymph nodes display longer telomeres than PB-$CD56^{dim}$ NK cells*

$CD56^{bright}$ NK cells display a more immature phenotype compared to $CD56^{dim}$ NK cells in many functional aspects, such as proliferative capacity, ability to produce cytokines and cytotoxic potential (50, 55, 70). To investigate whether $CD56^{bright}$ NK cells represent an earlier step of NK cell differentiation than $CD56^{dim}$ ones, telomere length in NK cell subsets isolated *ex vivo* from PB and LN were evaluated. The measurement of telomere length has been widely employed to assess the proliferative history of distinct cell subsets, among which are naïve and memory T cells (71-76). In most normal somatic cells, telomeres sequences are lost during DNA replication and therefore telomere length inversely correlates with cell age. Analysis of telomere length was performed in cooperation with Barbara Morandi and Guido Ferlazzo from the Department of Human Pathology, School of Medicine at the University of Messina in Italy.

As shown in Figure 2, sorted PB-CD56$^{dim}$ NK cells displayed significantly shorter telomere length than PB-CD56$^{bright}$ NK cells from the same donors ($p \leq 0.01$), with a mean telomere shortening of 15.3 % in the CD56$^{dim}$ compared to the CD56$^{bright}$ NK cells (Figure 2 B). For comparison, telomere length difference in naïve CD45RA$^{+}$ CD45RO$^{-}$ CD27$^{+}$ and memory CD45RA$^{-}$ CD45RO$^{+}$ CD4$^{+}$ T cells derived from PB of the same donors were calculated (Figure 2 C). It could be shown that telomere shortening in CD56$^{dim}$ compared to CD56$^{bright}$ NK cells (24.3 % and 15.4 %, respectively) correspond with the one observed in memory compared to naïve CD4$^{+}$ T cells. Notably, CD56$^{bright}$ NK cells displayed relative telomere length similar to naïve T cells, while CD56$^{dim}$ NK cells to memory T cells. In addition, comparative analysis of telomere length in PB-CD56$^{dim}$ NK cells and autologous LN-NK cells, which are predominantly CD56$^{bright}$, revealed that PB-CD56$^{dim}$ NK cells exhibited significantly shorter telomeres than LN-NK cells ($p \leq 0.04$), with a mean telomere shortening of 14.5 % in the PB-CD56$^{dim}$ compared to the LN-CD56$^{bright}$ NK cells (Figure 2 D). A similar degree of telomere length reduction was observed when LN-NK cells were sorted and cultured for as long as 3 weeks in the presence of 50ng/ml of IL-2, a cytokine that induces extensive NK cell proliferation (Figure 2 E). To exclude putative different ability in telomere maintenance between the two cell subsets being the reason for the observed differences in telomere length, telomerase activity was determined in highly purified CD56$^{bright}$ or CD56$^{dim}$ NK cells. Yet, the analysis of enzyme basal activity in PB-NK cells derived from three distinct donors did not show any difference between the two cell subset (data not shown) (77).

In total, these data indicate that CD56$^{bright}$ NK cells have undergone a significant smaller number of cell divisions *in vivo* as compared to CD56$^{dim}$ and might therefore represent an upstream developmental stage of NK cells.

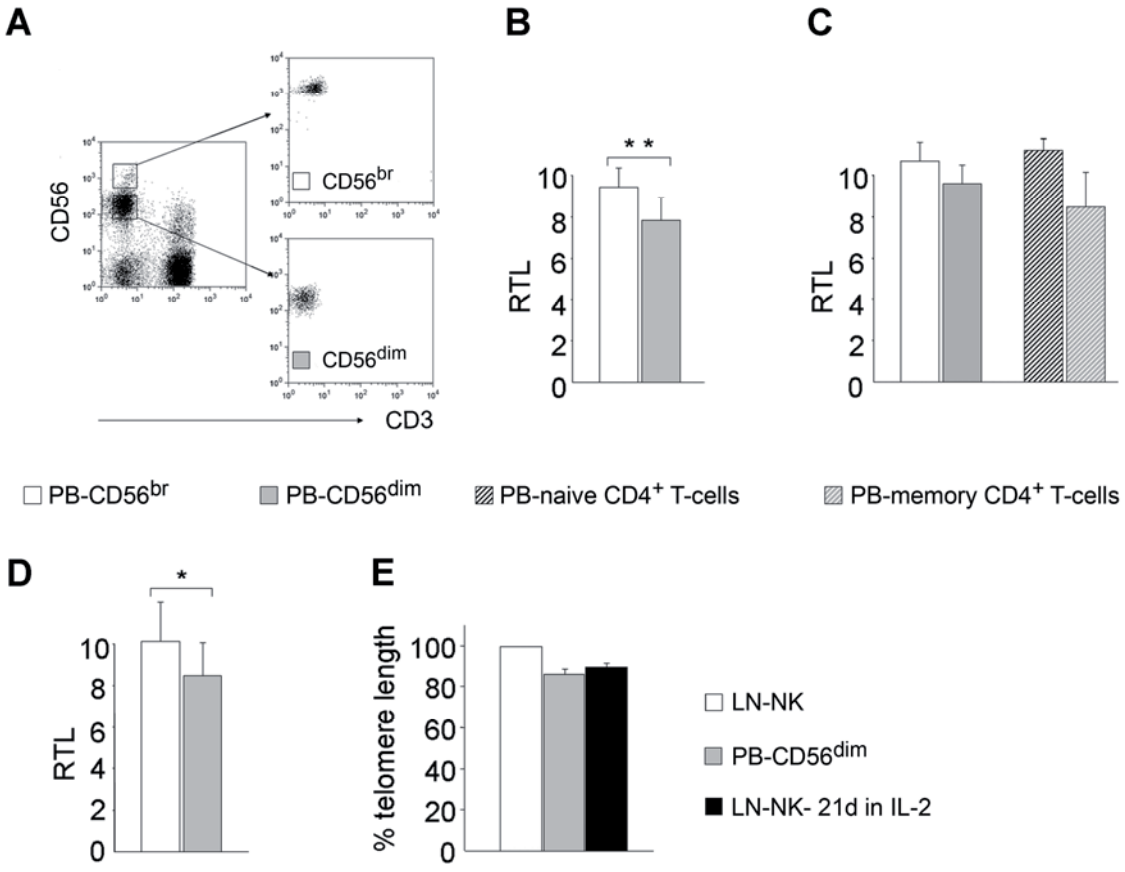


**Figure 2: PB-CD56$^{bright}$ and LN-NK cells display longer telomeres than PB-CD56$^{dim}$**

PB-CD56$^{bright}$, PB-CD56$^{dim}$ and LN-NK cells were sorted with high purity from different donors (A, one representative PB-NK cell sorting is depicted) by us and evaluated for their telomere length in cooperation with the Department of Human Pathology, University of Messina, Italy. (B) Relative telomere lengths (RTL) of PB-CD56$^{bright}$ and PB-CD56$^{dim}$ NK cells are shown. RTL was calculated as described in Materials and Methods. Mean RTL value ± SD of CD56$^{bright}$ NK cells (white bar) compared to the CD56$^{dim}$ NK cells (grey bar) of seven individual donors are shown ($_{**}$ $p \leq 0.01$, as calculated by paired Student's *t* test). (C) RTL values of PB-CD56$^{bright}$ (white bar) and CD56$^{dim}$ NK cells (grey bar) derived from two donors, included in (B), were compared to RTL values of autologous CD45RA$^{+}$ RO$^{-}$ CD27$^{+}$ naïve (black striped bar) and CD45RO$^{+}$ RA$^{-}$ memory (grey striped bar) CD4$^{+}$ T cells. Mean RTL value ± SD are depicted. (D) Telomere length of LN-NK cells (white bars) and PB-CD56$^{dim}$ NK cells (grey bars) were compared. Mean RTL value ± SD of 4 donors ($_{*}$ $p \leq 0.05$ by paired Student's *t* test). (E) Telomere length was analysed in total LN-derived NK cells sorted *ex vivo* (RTL set as 100%, white bars), in the same cells after 3 week culture with 100 IU/ml of IL-2 (black bar) and in autologous PB-CD56$^{dim}$ NK cells (grey bars). Mean RTL value ± SEM.

### *4.1.3 CD56$^{bright}$ NK cells acquire signature of CD56$^{dim}$ NK cells upon cytokine stimulation*

Although analysis of CD56$^{bright}$ versus CD56$^{dim}$ versus IL-2 activated CD56$^{dim}$ NK cell gene signature has been performed (26), no clear data exist concerning modulation of PB-CD56$^{bright}$ NK cell phenotype after activation and in particular during proliferation.

Since stimulation with cytokines results in activation and subsequent proliferation preferentially of CD56$^{bright}$ NK cells, it was tested, whether upon cytokine stimulation CD56$^{bright}$ can acquire the signature of CD56$^{dim}$ NK cells. To this aim, CFSE-labelled PB-CD56$^{bright}$ and CD56$^{dim}$ NK cells were cultured with IL-2, IL-12 or IL-15 for 5 days to induce proliferation, and modulation of markers which are differentially expressed between the two subsets, i.e. IL7Rα, c-kit, CXCR3, CCR7, CD62L (mainly expressed on CD56$^{bright}$ NK cells), granzymeA and granzymeB (almost confined to CD56$^{dim}$ NK cells) was investigated. After 5 days of cytokine stimulation, changes in expression of each molecule was assessed comparing its mean fluorescence intensity (MFI) among cells which have undergone extensive proliferation, as determined by the loss of CFSE intensity, to the MFI among resting cells cultured in medium (Figure 3, A and B, first column) for the same period of time. Notably, expression of each molecule analysed *ex vivo* did not significantly change after 5 days of culture in medium alone, i.e. without cytokines (data not shown).

As shown in Figure 3 A, CD56$^{bright}$ NK cells down-regulated surface expression of IL7Rα, c-kit, CXCR3 and CCR7 after IL-2, IL-12 (and IL-15, data not shown) culture in stimulated cells compared to resting ones kept in medium. Remarkably, down-regulation of cell surface expression of these molecules was not necessarily related to proliferation, as MFI decreased already in non-dividing cells cultured in the presence of cytokines when compared to the ones kept in medium only. CD62L expression on CD56$^{bright}$ NK cells was strongly down regulated after IL-2 (and IL-15, data not shown) stimulation, while even up regulated by IL-12 independently of proliferation. Although entering the cell cycle, CD56$^{dim}$ NK cells did not acquire any of these molecules under the same culture conditions, with the exception of CD62L, which was partly induced on CD56$^{dim}$ NK cells after IL-12 stimulation (Figure 3 B). Intracellular staining of cytokine stimulated CD56$^{bright}$ NK cells revealed that they acquired cytolytic molecules such as granzymeA and granzymeB upon all stimulating conditions (Figure 3 A, and data not shown for IL-15), while the same molecules remained rather stable or were even up-regulated in CD56$^{dim}$ NK cells (Figure 3 B).

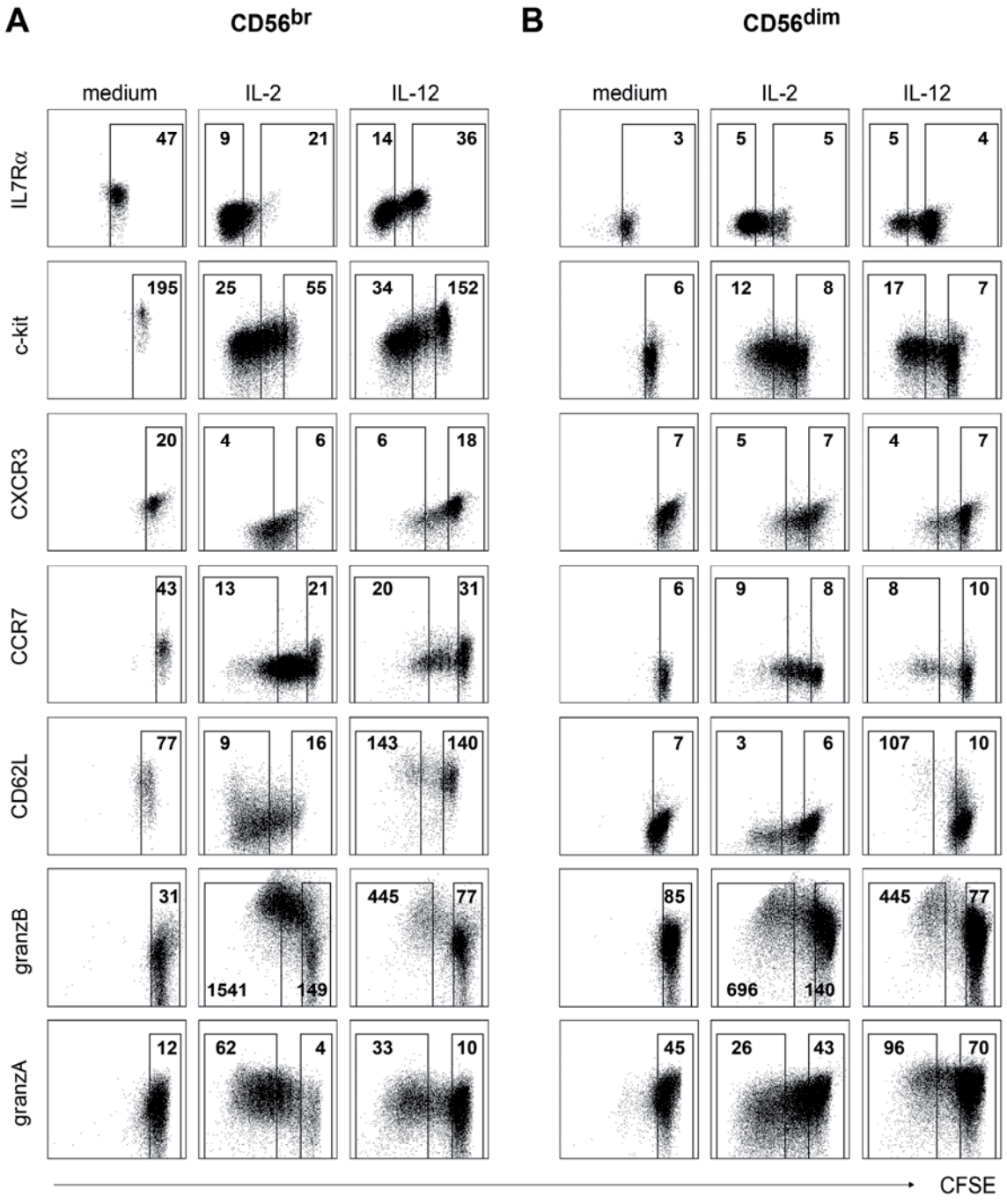


**Figure 3: PB-CD56$^{bright}$ NK cells acquire signature of CD56$^{dim}$ NK cells upon cytokine activation**

Sorted CD56$^{bright}$ (A) or CD56$^{dim}$ NK cells (B) were CFSE-labelled and cultured in the presence of medium, IL-2 or IL-12. Because a fraction of CD56$^{dim}$ NK cells can express CD62L, for this experiment (fifth row), CD56$^{dim}$ NK cells were negatively sorted for CD62L. At day 5, modulation of the indicated surface or intracellular molecule was evaluated comparing mean fluorescence intensity (MFI) among resting (right gate) and proliferating (left gate) cells, as depicted in each dot plot.

Concerning the expression of CD56, no down-regulation was expected on CD56$^{bright}$ NK cells, since it is known that high levels of CD56 are induced and maintained in all NK cells during activation. Nevertheless, LN-NK cells that have been stimulated with IL-2 down regulated their level of CD56 expression following IL-2 withdrawal, while KIR expression was maintained (data not shown).

### *4.1.4 PB-CD56$^{bright}$ NK cells can acquire KIR and CD16 expression upon cytokine activation*

Some reports have shown, that CD16, which is expressed by the majority of PB-CD56$^{dim}$ NK cells, can be down regulated (58, 59), suggesting that CD56$^{bright}$ CD16$^{-}$ NK cells might represent activated CD56$^{dim}$ NK cells. In contrast, down-regulation of KIR expression from PB-CD56$^{dim}$ KIR$^{+}$ NK cells has never been reported so far.

In order to investigate modulation of CD16 expression by cytokine stimulation, CD56$^{bright}$ CD16$^{-}$ and CD56$^{dim}$ CD16$^{+}$ NK cells were sorted, CFSE labelled and cultured as described in Figure 3. Analysis of CD16 surface expression at day 5 of culture showed that significant CD16 expression occurs on the majority of CD56$^{bright}$ CD16$^{-}$ NK cells after IL-2 (and IL-15, data not shown) stimulation while IL-12 was less efficient (Figure 4, upper panel). As previously reported (58), partial loss of CD16 expression was observed on CD56$^{dim}$ CD16$^{+}$ NK cells in the presence of IL-12, as well as when cells were left for 5 days in medium alone, although they have been previously sorted with very high purity for CD16 expression (Figure 4, lower panel).

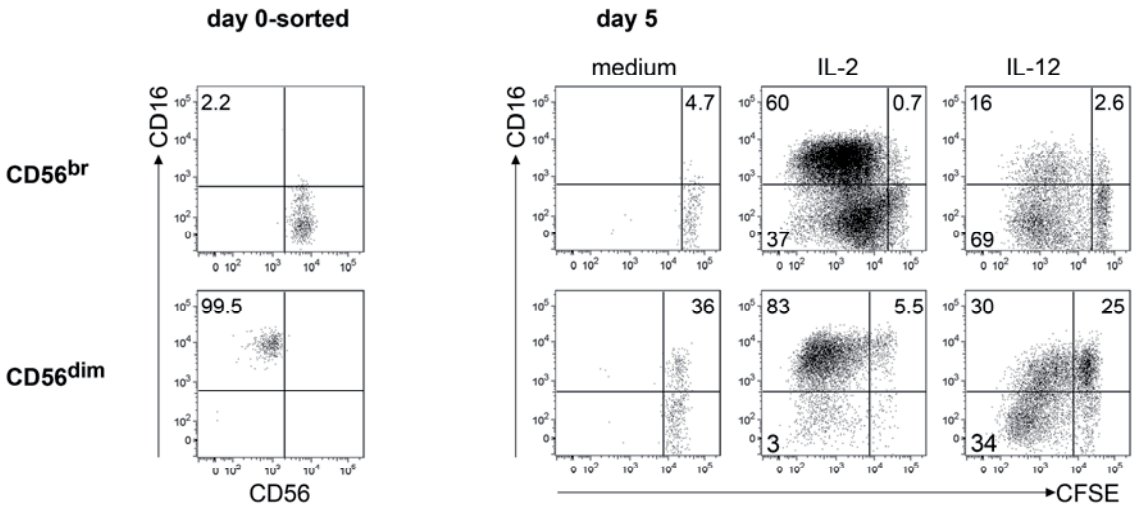


**Figure 4: PB-CD56$^{bright}$ NK cells up-regulate CD16 expression upon cytokine activation.**

CD56$^{bright}$ CD16$^{-}$ and CD56$^{dim}$ CD16$^{+}$ NK cells were sorted, CFSE-labelled and cultured in the presence or in the absence of IL-2 or IL-12. At day 5, modulation of surface CD16 expression was evaluated after staining with anti-CD16 antibody. Percentages of CD16$^{+}$ cells are indicated in each dot plot. One representative experiment out of five is shown.

To evaluate alterations of KIR expression in the distinct PB-NK cell subsets, NK cells were sorted with high purity after staining with a combination of all available anti-KIR (anti-KIR2DL2/S2/L3, KIR2DL1/S1, -KIR3DL1/S1/L2) antibodies. CFSE-labelled CD56$^{bright}$ KIR$^{-}$, CD56$^{dim}$ KIR$^{+}$ and CD56$^{dim}$ KIR$^{-}$ NK cells were cultured in the presence of IL-2, IL-12 or IL-15 and analysed for KIR surface expression at day 5. As shown in Figure 5 A, both CD56$^{bright}$ KIR$^{-}$ (top row) and CD56$^{dim}$ KIR$^{-}$ NK cells (middle row) exhibited de-novo expression of KIR on a significant proportion of cells in response to IL-2 (and IL-15, data not shown) and in lower percentage in response to IL-12. In contrast, none of these stimuli was able to down-regulate KIR expression on sorted CD56$^{dim}$ KIR$^{+}$ NK cells, which was even up-regulated after IL-2 stimulation (Figure 5 A, bottom row). As previously shown, CD56$^{bright}$ NK cells displayed a higher proliferative response both to IL-2, IL-15 and IL-12 compared to total CD56$^{dim}$ NK cells.

Nonetheless, the comparison of the proliferative ability at day 5 of $CD56^{bright}$ in response to 50ng/ml of IL-2 (percentage of mean proliferation ± SEM = 92.5% ± 1.6) with the one of $CD56^{dim}$ $KIR^-$ (67.7% ± 9.5) or $KIR^+$ NK cells (26.4% ± 5.8) showed that $CD56^{bright}$ proliferate slightly more than $CD56^{dim}$ $KIR^-$ NK cells ($p \leq 0.05$), while $CD56^{dim}$ $KIR^+$ proliferate significantly less compared not only to $CD56^{bright}$ ($p \leq 0.002$) but also to $KIR^-$ NK cells ($p \leq 0.015$) (Figure 5 A), suggesting that expression of KIR might correlate with a terminally differentiated phenotype. Outstandingly, the lower proliferative capacity of $KIR^+$ NK cells excludes the possibility that rare contaminating $KIR^+$ NK cells could overgrow $CD56^{bright}$ and $CD56^{dim}$ $KIR^-$ NK cells and be responsible for detection of KIR expression among $KIR^-$ NK cells.

In order to investigate more in detail which KIR could be induced on the surface of $CD56^{bright}$ or $CD56^{dim}$ $KIR^-$ NK cells, NK cell subsets were sorted as previously described and analysed for single KIR expression after 5 days of culture in IL-2. As shown in Figure 5 B, each KIR expressed *ex vivo* on $CD56^{dim}$ $KIR^+$ NK cells (left column) could be induced on the surface of $CD56^{bright}$ $KIR^-$ or $CD56^{dim}$ $KIR^-$ NK cells (right columns) by cytokine stimulation. Next, in donors in which staining of single KIR was performed, mRNA expression of KIR2D and KIR3D ORF (see materials and methods) and of their activating or inhibitory counterparts was analysed either directly after sorting or at day 5 of IL-2 culture. RT-PCR was performed in cooperation with Michela Falco from the Institute Giannina Gaslini, Genoa, Italy. As shown in Figure 5 C, IL-2 stimulation induced mRNA expression of both inhibitory and activating KIR both in $CD56^{bright}$ and $CD56^{dim}$ $KIR^-$ NK cells. The signal detectable in $CD56^{dim}$ $KIR^-$ NK cells for all KIR ORF mRNA at day 0 after sorting seems to be mainly related to activating rather than inhibitory KIR. Indeed, this could be due to the use of primers recognizing not only KIR2DL2/S2/L3, KIR2DL1/S1, -KIR3DL1/S1/L2 (stained for FACS sorting) but also KIR2DS3/S4/S5 for which NK cells could not be negatively sorted due to the lack of specific antibodies.

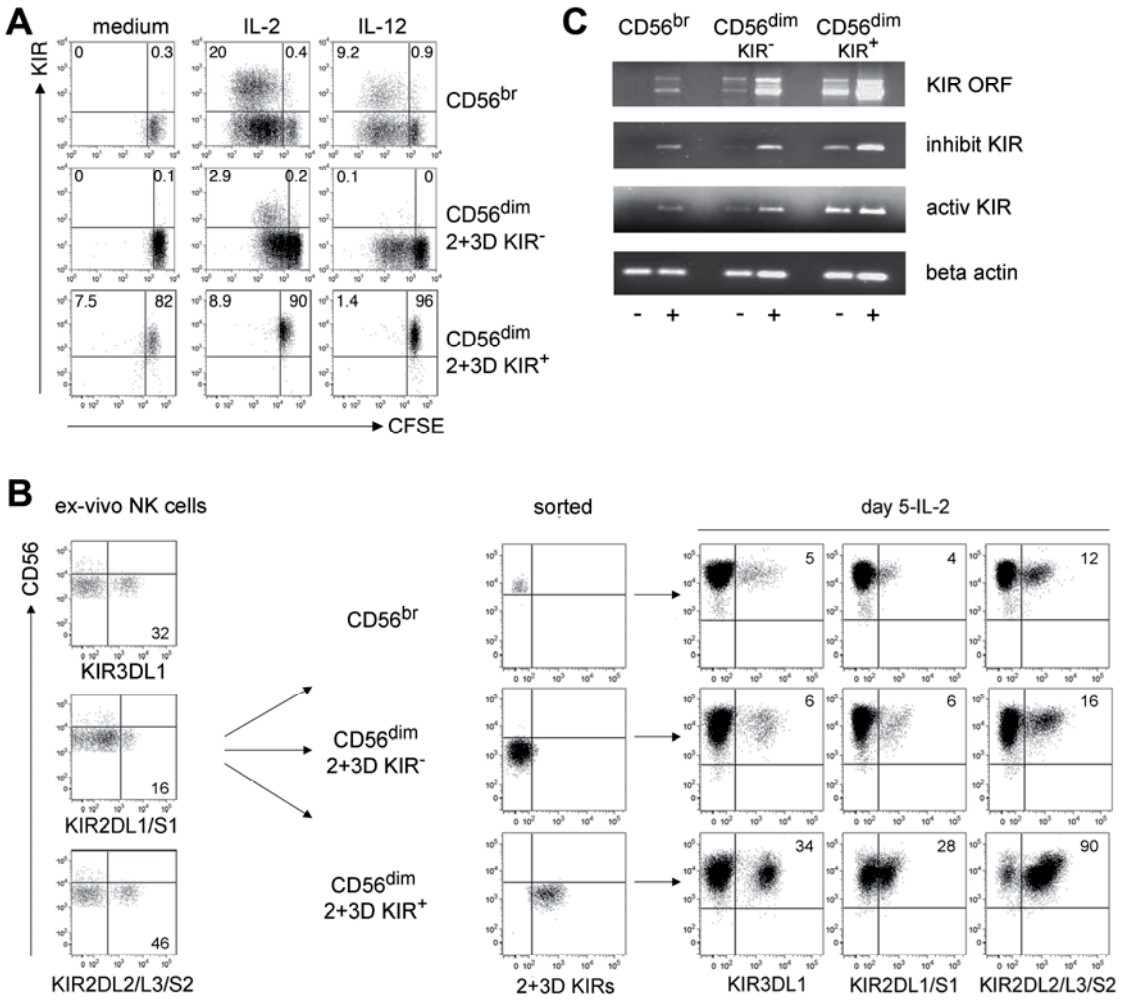


**Figure 5: PB-CD56$^{bright}$ and CD56$^{dim}$ KIR$^{-}$ NK cells express KIR upon cytokine activation.**

Sorted NK cell subsets were CFSE-labelled and cultured in the presence or in the absence of IL-2 or IL-12 for 5 days. At day 5, modulation of KIR expression was evaluated. (A) CD56$^{bright}$, CD56$^{dim}$ KIR$^{-}$ and CD56$^{dim}$ KIR$^{+}$ NK cells were stained with a combination of antibodies specific for 2D KIRs and 3D KIRs. One representative experiment out of six is shown. (B) *Ex vivo* expression of single KIR after staining with antibodies directed against KIR2DL2/L3/S2, KIR2DL1/S1 or KIR3DL1 (left column) on total NK cells was evaluated. CD56$^{bright}$, CD56$^{dim}$ 2+3D KIR$^{-}$ and CD56$^{dim}$ 2+3D KIR$^{+}$ NK cells were sorted. Purity check of the sorted subsets from one representative experiment is shown (middle column). After IL-2 culture, induction of each single KIR on the sorted NK cell subsets (right columns) was measured. One representative experiment out of three is shown. (C) Total RNA was isolated from sorted CD56$^{bright}$, CD56$^{dim}$ 2+3D KIR$^{-}$ and CD56$^{dim}$ 2+3D KIR$^{+}$ NK cell subsets derived from the same donor shown in (B). PCR products obtained using sets of primers specific for KIR3D (first lane, upper band) and KIR2D (first lane, lower band) ORF transcripts, and their inhibitory (second lane) or activating (third lane) counterparts are shown. RT-PCR of a beta actin segment (fourth

lane) was used as positive transcription control. Analysis was performed directly after sorting or after 5 day culture in IL-2. One representative experiment out of two is shown.

### *4.1.5 Secondary lymphoid organs can be site of NK cell maturation*

It has recently been suggested that SLO might be sites of NK cell differentiation (50, 52). If this was indeed the case, NK cells leaving LN should be different from NK cells resident in LN. To this aim, in cooperation with Guido Ferlazzo, NK cells isolated from the efferent lymph system (i.e. thoracic duct) and from autologous LN have been analysed in parallel. Consistent with previous reports (49, 50), NK cells located in non reactive LN displayed low or no KIR and CD16 expression (Figure 6 A). Alternatively, a significant fraction of NK cells collected from the efferent lymph of the thoracic duct expressed KIR and CD16, although the latter to a lower extent than their blood counterpart (Figure 6 A). These data cannot rule out the possibility that a small percentage of $CD56^{dim}$ NK cells expressing KIR and CD16 enters the LN, expands in situ and then leaves LN via efferent lymph. Nonetheless, considering the lower proliferative ability and the chemokine receptor expression of $CD56^{dim}$ $CD16^{+}$ $KIR^{+}$ NK cells, the hypothesis that NK cells can acquire *de novo* expression of relevant functional molecules in LN and then circulate to PB through the efferent lymph can be favoured.

The observation that NK cells emigrating from, but not resident in LN, express KIR and CD16 (Figure 6 A), raised the question whether KIR and CD16 acquisition occurs only in reactive or as well in non-reactive LNs. Therefore, NK cells isolated from 28 LN of 14 individual donors were analysed. As shown in Figure 6, a significant percentage of NK cells expressing KIR was detectable only in reactive LN characterized by paracortical/follicular hyperplasia (mean percentage ± SEM: 7.3 ± 0.3, Figure 6 C), which is branded by the presence of secondary follicules and lymphocyte proliferation (Figure 6 B, iii-vi). NK cells isolated from non-reactive LN (Figure 6 B, i-ii) or LN with sinus hyperplasia (characterized by an increased number of macrophages) showed low or no expression of KIR (mean percentage ± SEM: 1.75 ± 0.2, Figure 6 C). In addition, CD16 expression correlated with LN-paracortical/follicular hyperplasia (data not shown) (77). Because of the strong association of KIR and CD16 expression with LN-paracortical/follicular hyperplasia ($p \leq 0.002$), it could be hypothesized that KIR expression in LN NK cells might represent a *de novo* induction of these molecules occurring on LN resident $CD56^{bright}$ NK cells in the course of an inflammatory immune response, were different cytokines (e. g. IL-15, IL-12 and IL-2) are present.

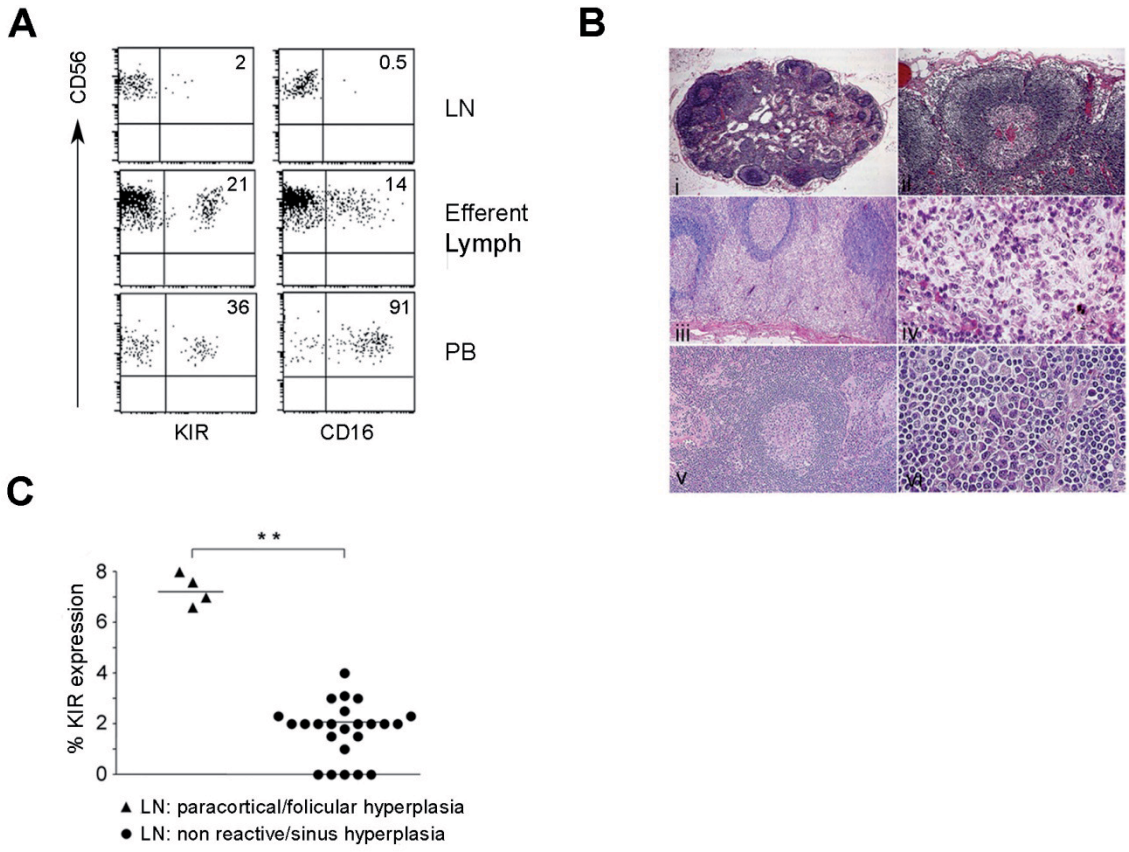


**Figure 6: NK cells from efferent lymph and from LN with paracortical/follicular hyperplasia express KIR and CD16.**

This experiment was performed by Guido Ferlazzo, University of Messina, Italy.

(A) Mononuclear cells were isolated from efferent lymph (i.e. thoracic duct), LN and PB of the same donor. CD16 and KIR expression was analysed gating on NK cells. One representative experiment out of two is shown. (B) Sections of paraffin-embedded tissue of 28 LN were stained with Hematoxylin-Eosin. 3 LN that are representative of distinct inflammatory conditions as detected by histological analysis out of the 28 analysed are shown. (i): non reactive LN, magnification 5X. (ii): detail of a follicle shown in (i), magnification 40X. (iii): LN paracortical hyperplasia: manifest enlargement of the paracortical area between capsula and follicles, magnification 5X. (iv): detail of the paracortical area shown in (iii), magnification 40X. (v): LN follicular hyperplasia, magnification 5X. (vi): detail of the follicular hyperplasia showing the presence of several plasma cells. (C) Percentage of KIR expressing NK cells isolated from LN exhibiting paracortical or follicular hyperplasia or combination of the 2 (triangles) or from LN displaying no reactivity or sinus hyperplasia (circles), according to the histological analysis. The median value for each group is depicted ($p \leq 0.0018$ by Mann-Whitney test). In order to correlate LN inflammatory status and NK cell KIR or CD16 expression, LN were incised immediately after removal and split into two sagittal parts, one of which was paraffin embedded in order to perform histology, while the other was processed for single cell isolation and analysed by flow cytometry.

Altogether, the results of this first part suggest that CD56$^{bright}$ and CD56$^{dim}$ NK cells correspond to sequential steps of differentiation, i.e. CD56$^{bright}$ NK cell are precursors and can give rise to CD56$^{dim}$ cells and support the hypothesis that secondary lymphoid organs can be sites of NK cell final maturation and self-tolerance acquisition during an immune reaction.

## 4.2 Education of hyporesponsive NK cells by cytokines

In the first part of this study it was shown that under the influence of cytokines CD56$^{bright}$ can differentiate *in vitro* and *in vivo* into cells similar to CD56$^{dim}$ ones. During this process they acquire on the one hand cytotoxic effector molecules such as granzymes (Figure 3) and perforin (data not shown) and on the other hand inhibitory receptors, i.e. KIRs (Figure 5 and 6), a prerequisite for the discrimination of self from non self and thereby for tolerance towards self.

NK cell tolerance to self is mediated via engagement of inhibitory receptors by cognate MHC molecules. In humans, inhibitory receptors include KIRs, exclusively expressed by CD56$^{dim}$, and the CD94/NKG2A heterodimer, present on all CD56$^{bright}$ and on a subset of CD56$^{dim}$ NK cells. It has been shown that expression of at least one self-specific inhibitory receptor is critical for NK cell education to achieve functional competence. Thus, NK cells expressing self-MHC specific inhibitory receptors are responsive to activating stimuli while those lacking such receptors (KIR$^{-}$NKG2A$^{-}$) are hyporesponsive (37-40). Nevertheless, the mechanisms underlying NK cell education are still poorly understood. In the second part of this thesis it was investigated whether cytokine induced expression of self specific inhibitory receptors results in education of hyporesponsive NK cells to become competent and self tolerant.

### *4.2.1 Cytokines induce stable expression of inhibitory receptors on hyporesponsive CD56$^{dim}$ KIR$^{-}$ NKG2A$^{-}$ NK cells*

CD56$^{dim}$ NK cells that do not express any inhibitory receptor specific for MHC-class I, have been shown to be hyporesponsive after stimulation with MHC class I negative targets (39, 40, 78). Nonetheless, it is still debated whether this NK cell subset maintains its phenotype during inflammation, when receiving different types of signals, including cytokines.

Accordingly, it was investigated whether cytokines are able and sufficient to induce expression of inhibitory receptors not only in $CD56^{bright}$ and $CD56^{dim}$ $KIR^-$ NK cells, as shown in Figure 5, but also in $CD56^{dim}$ $KIR^-$ $NKG2A^-$ ones.

Stimulation of sorted $CD56^{dim}$ $KIR^-$ $NKG2A^-$ NK cells with IL-2, IL-15 (Figure 7 A), IL-12 or IL-12 plus IL-18 (data not shown) resulted in KIR induction on a fraction of NK cells earliest at day 2. *De novo* expression of KIRs was maintained until day 16 and progressively decreased until day 26. Moreover, induction of KIR expression was not restricted to $KIR^-$ $NKG2A^-$ cells but could be also observed in cells, which were already positive for one or more KIRs (Figure 7 B). In contrast to KIR expression, NKG2A expression was induced already at day 1 on the majority of $KIR^-$ $NKG2A^-$ NK cells and remained stable for the duration of the culture (Figure 7 A) with only small changes depending upon the activation status of the cells. These data show that cytokines alone are sufficient to induce expression of KIRs and NKG2A on hyporesponsive $KIR^-$ $NKG2A^-$ NK cells.

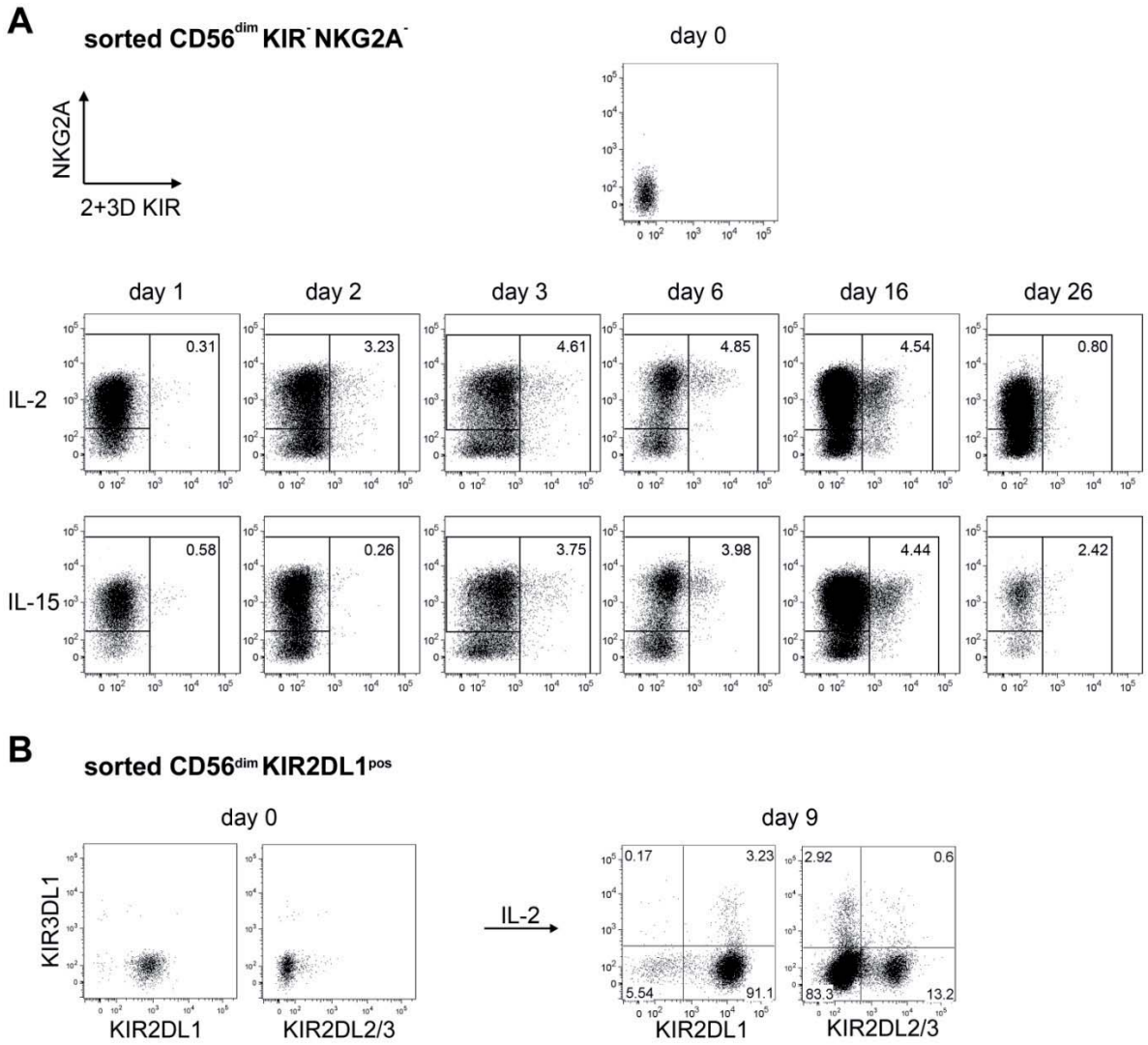


**Figure 7: Cytokines induce KIR and NKG2A expression on hyporesponsive KIR$^-$ NKG2A$^-$ NK cells**

(A) Sorted CD56$^{dim}$ KIR$^-$ NKG2A$^-$ NK cells were cultivated in the presence of IL-2 or IL-15 and analysed for KIR and NKG2A expression after staining with a combination of antibodies specific for 2D KIRs and 3D KIRs and anti-NKG2A at different time points. Percentage of KIR induction is shown. One representative experiment out of three is depicted. (B) Sorted CD56$^{dim}$ KIR2DL1$^+$ KIR2DL2/3$^-$ KIR3DL1$^-$ NK cells were cultivated in the presence of IL-2 and analysed for KIR expression at day 9 of culture. Percentage of KIR induction in one representative experiment out of three is shown.

As shown in Figure 7, KIR expression slowly disappeared over time. This finding implies two possible explanations: either $KIR^-$ NK cells overgrow the $KIR^+$ ones, as it has been previously demonstrated that $KIR^-$ NK cells have a higher proliferative capacity than $KIR^+$ cells (Figure 5) (78), or KIRs are down-regulated. In order to rule out the last possibility, $KIR^-$ $NKG2A^-$ NK cells which after IL-2 culture expressed a single KIR (in this case KIR3DL1) were again sorted and cultivated in IL-2 for additional 12 days (Figure 8). In parallel, *ex vivo* sorted $KIR3DL1^+$ NK cells were also cultivated in IL-2 for the same period. As shown in Figure 8, KIR3DL1 expression induced by a first round of IL-2 stimulation remained stable on the majority of cells during the second round of IL-2 culture (Figure 8), once $KIR3DL1^+$ cells were again purified. However, within $KIR3DL1^+$ cells generated *in vitro*, a slightly higher percentage of cells appeared to lose KIR3DL1 expression as compared to the *ex vivo* $KIR3DL1^+$ sorted ones, indicating that KIRs acquired *in vivo* are probably more stable than those acquired *in vitro*. Remarkably, NK cells which did not acquire any KIR expression in the first 12 days of IL-2 or IL-15 stimulation failed to do so in the following 12 days of culture (Figure 8), while NKG2A could be still significantly induced (data not shown).

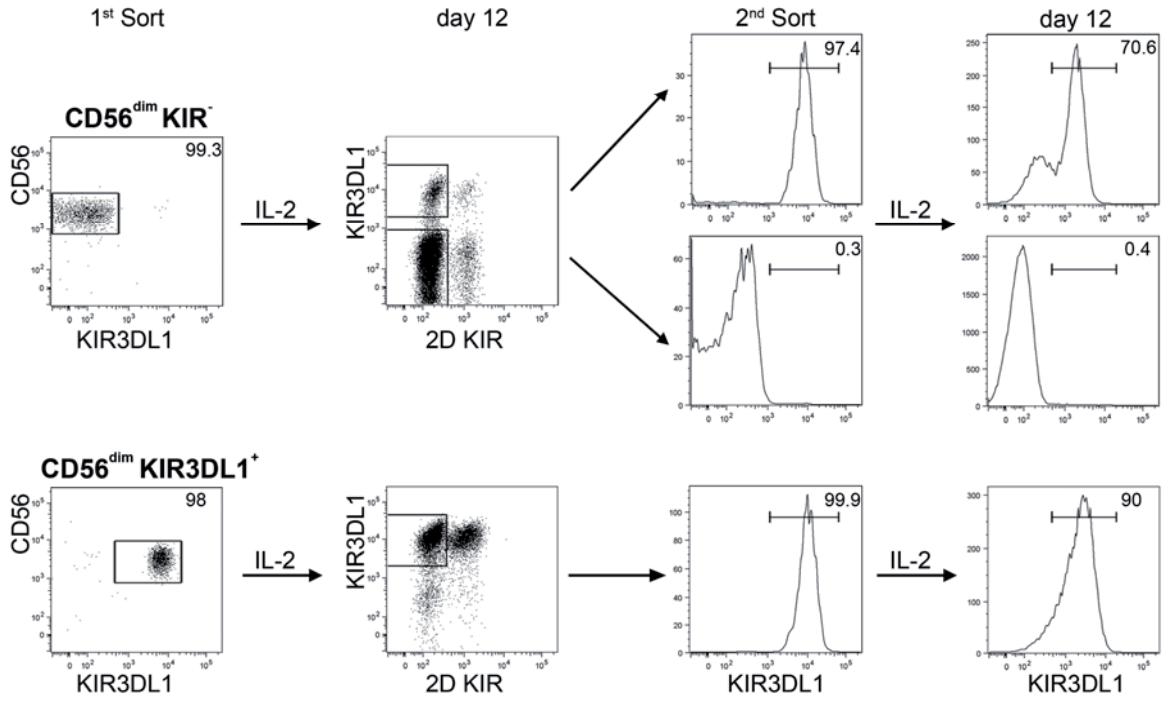


**Figure 8: Cytokines induce stable KIR3DL1 expression on a subset of KIR$^-$ NKG2A$^-$ NK cells**

CD56$^{dim}$ KIR3DL1$^-$ 2D KIR$^-$ NKG2A$^-$ NK cells were sorted and cultivated for 12 days in the presence of IL-2 to induce expression of KIR3DL1. At day 12, NK cells were stained for KIR3DL1 and a combination of antibodies specific for 2D KIRs. KIR3DL1$^+$ 2D KIR$^-$ and KIR3DL1$^-$ 2D KIR$^-$ NKG2A$^-$ cells were re-sorted (2$^{nd}$ Sort) and cultivated for additional 12 days with IL-2. As control, *ex vivo* isolated KIR3DL1$^+$ NK cells were also cultivated and re-sorted. One representative experiment out of four is depicted.

Overall, these data demonstrate that cytokines are sufficient to induce a rather stable expression of KIRs on a subpopulation of KIR$^-$ NKG2A$^-$ NK cells and of NKG2A on the majority of cells.

### *4.2.2 Acquisition of self specific KIRs after cytokine stimulation confers competence to CD56$^{dim}$KIR$^-$NKG2A$^-$ hyporesponsive NK cells*

Since *ex vivo* expression of self-specific inhibitory receptors correlates to NK cell functional competence (39, 40), it was analysed whether acquisition of KIR expression after cytokine stimulation confers competence to CD56$^{dim}$ KIR$^-$ NKG2A$^-$ hyporesponsive NK cells and if engagement of self-MHC is required.

To answer these questions, KIR3DL1 expression was induced in $CD56^{dim}$ $KIR^-$ $NKG2A^-$ NK cells from HLA-Bw4 or HLA-Bw6 individuals, which do or do not express the cognate MHC ligand for KIR3DL1, respectively. After 12 days of IL-2 culture, 13.5 ± 4% of $KIR^-$ $NKG2A^-$ cells expressed at least one KIR. The percentage of $KIR3DL1^+$ NK cells was 3.3 ± 1.7% among HLA-Bw4 and 1.9 ± 1% in HLA-Bw6 individuals ($p \leq 0.15$, as calculated by Wilcoxon test), thus showing that induction of a certain KIR does not require the presence of the cognate MHC ligand. Cells, which expressed exclusively KIR3DL1, were compared to cells remaining $KIR^-$ $NKG2A^-$ for their cytotoxic competence by measuring surface expression of CD107a, a marker for degranulation, after stimulation with the MHC class I negative tumour cell line K562. As shown in Figure 9 A, NK cells that acquired KIR3DL1 in HLA-Bw4 individuals displayed significantly higher CD107a expression than NK cells, which remained $KIR^-$ $NKG2A^-$, suggesting that acquisition of KIRs correlates with functional competence. In order to confirm that only acquisition of self specific KIRs correlated with induction of functional competence in hyporesponsive NK cells, the same experiment was performed analyzing NK cells derived from HLA-Bw6 individuals, which do not express the cognate ligand for KIR3DL1. In contrast to what has been observed in HLA-Bw4 individuals, single $KIR3DL1^+$ NK cells generated from HLA-Bw6 individuals after IL-2 culture remained as hyporesponsive as $KIR^-$ $NKG2A^-$ NK cells (Figure 9 A). These data demonstrate that cytokine stimulation results in education of hyporesponsive $KIR^-$ $NKG2A^-$ NK cells by inducing self-MHC specific KIRs, as it occurs only in HLA-Bw4 but not in HLA-Bw6 individuals. Moreover, this finding supports the concept that KIR engagement with the corresponding self-MHC ligand is required for this process.

As stimulation with IL-2 induces expression not only of inhibitory receptors but also of several molecules involved in NK cell activation and cytotoxicity, expression levels of activating receptors namely NKp30, NKp46, NKG2D, of CD3ζ chain, and of lytic molecules, namely perforin and granzymes in $KIR3DL1^+$ and $KIR^-$ $NKG2A^-$ NK cells from HLA-Bw4 individuals generated after IL-2 culture were compared. As shown in Figure 9 B, both NK cell subsets displayed comparable expression levels of each molecule analysed, suggesting that none of them is involved in induction of NK cell competence by cytokines.

As demonstrated in Figure 9 A, expression of self-MHC specific KIRs induced by cytokine stimulation confers cytotoxic competence to previously hyporesponsive NK cells. Since hyporesponsiveness is the mechanism that enables $KIR^-$ $NKG2A^-$ cells to keep tolerance towards self, it had to be investigated whether cytokine stimulation would lead to the development of potentially autoreactive NK cells. To this end, the cytotoxic potential of $KIR3DL1^+$ NK cells generated from HLA-Bw4 individuals after cytokine stimulation against 721.221 transfectants expressing or not expressing the MHC class I ligand for KIR3DL1, i.e. HLA-Bw4 was evaluated. As already observed for stimulation using K562 as target cells (Figure 9 A), cytokine generated $KIR3DL1^+$ NK cells displayed higher CD107a expression after stimulation with 721.221 targets than cells remaining $KIR^-$ $NKG2A^-$ (data not shown). Notably, lysis of 721.221 could be significantly inhibited by the expression of HLA-Bw4, but not of HLA-Bw6, demonstrating that cytokine induced KIRs are functional and render $KIR^+$ NK cells tolerant versus cells that express the cognate self-MHC ligand (Figure 9 C).

These data show that induction of competence correlates with acquisition of functional inhibitory receptors specific for self-MHC and therefore should not result in autoreactivity, since hyporesponsiveness is replaced by MHC-dependent inhibition.

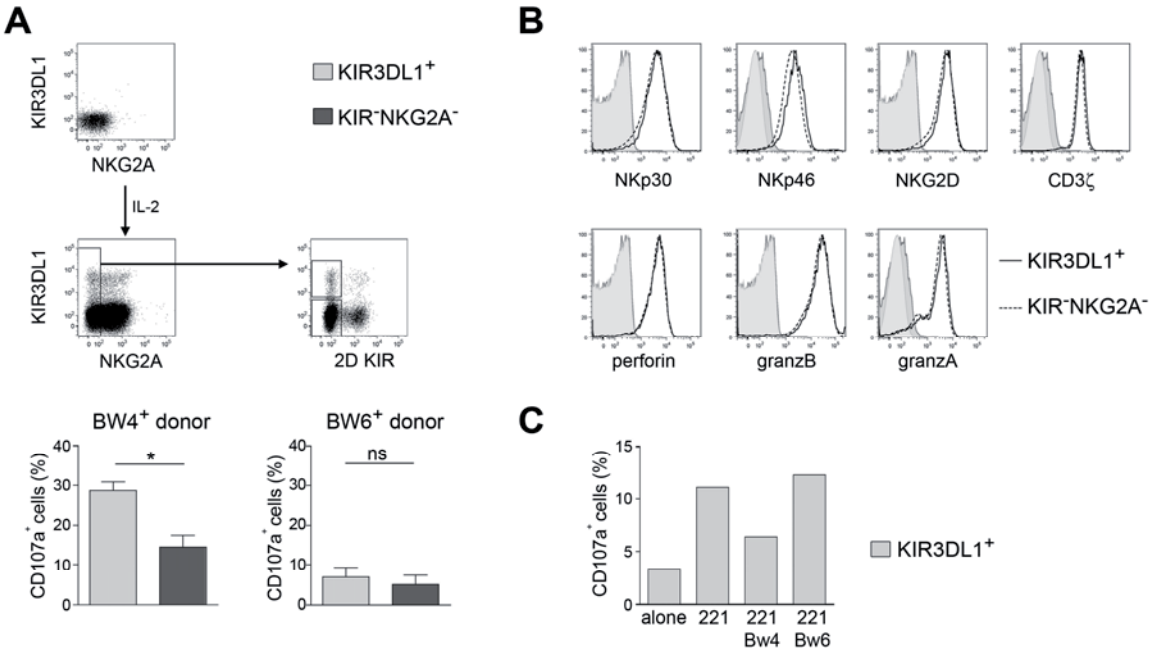


**Figure 9: Acquisition of self-MHC specific KIRs confers cytotoxic competence to hyporesponsive NK cells.**

(A) IL-2 cultured CD56$^{dim}$ KIR3DL1$^{-}$ 2D KIR$^{-}$ NKG2A$^{-}$ NK cells were stimulated with K562, stained for KIR3DL1 and a combination of antibodies specific for 2D KIRs and CD107a expression was measured after gating on KIR3DL1$^{+}$ 2D KIR$^{-}$ NKG2A$^{-}$ or KIR3DL1$^{-}$ 2D KIR$^{-}$ NKG2A$^{-}$ NK cells. Mean values ± SEM of six HLA-Bw4 (left) and six HLA-Bw6 individuals (right) are shown. (B) Expression of the indicated molecules and their isotype control was analysed in IL-2 generated KIR3DL1$^{+}$ 2D KIR$^{-}$ NKG2A$^{-}$ (solid line and gray-filled histogram) or KIR3DL1$^{-}$ 2D KIR$^{-}$ NKG2A$^{-}$ NK cells (dashed line and light gray-filled histogram). (C) CD107a expression after stimulation with the 721.221 parent cell line or 721.221 transfectants expressing HLA-B51 (HLA-Bw4) or HLA-B7 (HLA-Bw6) was assessed gating on KIR3DL1$^{+}$ 2D KIR$^{-}$ NK cells generated after IL-2 culture. Values derived from one representative experiment out of three are shown. All p values were calculated by Wilcoxon test. * indicates $p<0.05$.

Altogether, the data shown in the second part of my thesis clearly demonstrate that competent NK cells can be generated by cytokine stimulation, suggesting that NK cell education might not only be an early event which takes place during NK cell development but might also occur in the periphery during an immune response.

## 4.3 CD62L expression identifies a subset of polyfunctional CD56$^{dim}$ NK cells

In the first two parts of this thesis it was shown that under the influence of cytokines CD56$^{bright}$ can differentiate into CD56$^{dim}$ NK cells and that cytokine induced acquisition of inhibitory receptors results in the generation of competent and self-tolerant NK cells. In the third part, the heterogeneity of the CD56$^{dim}$ NK cell compartment with regard to expression of other markers than inhibitory receptors and with respect to functional properties associated to expression of these molecules has been elucidated.

As described already before, human NK cells comprise two main subsets that differ in function, phenotype and tissue localization, the CD56$^{bright}$ and the CD56$^{dim}$ NK cells (3.1). However, CD56$^{dim}$ NK cells represent as well a heterogeneous population concerning the expression of several markers, such as inhibitory receptors (3.1 and 3.2), CD62L or CD27. The aim of this part was to analyse how the expression of CD62L and of other markers correlates to different NK cell functions such as proliferation, cytokine production and cytotoxicity in order to identify intermediate stages of NK cell maturation and to better define the differentiation history of human NK cells.

### *4.3.1 CD56$^{bright}$ and CD56$^{dim}$ CD62L$^{+}$ NK cells display similar proliferative capacity in vivo and in vitro*

CD56$^{bright}$ NK cells have been shown to exhibit a much higher capacity to proliferate after *in vitro* stimulation with cytokines or dendritic cells (DC) than CD56$^{dim}$ NK cells (Figure 3-5) (19, 30, 70). Since CD56$^{dim}$ cells are not a homogenous population concerning the expression of several surface markers including CD62L (Figure 1), we first compared the proliferative ability of CD56$^{bright}$, CD56$^{dim}$ CD62L$^{+}$ and CD56$^{dim}$ CD62L$^{-}$ subsets after stimulation with cytokines or PB-derived myeloid (mDC). Activation with IL-2 (Figure 10 A and B), IL-15, IL-12 or DC (Figure 10 A) resulted in higher proliferation of CD56$^{bright}$ and CD56$^{dim}$ CD62L$^{+}$ cells as compared to CD56$^{dim}$ CD62L$^{-}$ ones. CD56$^{bright}$ NK cells proliferated slightly more than CD56$^{dim}$ CD62L$^{+}$ ones only at high concentrations of IL-2 (Figure 10 B), while the difference in proliferation efficiency between the two subsets became more striking at very low concentrations of IL-2 (Figure 10 C).

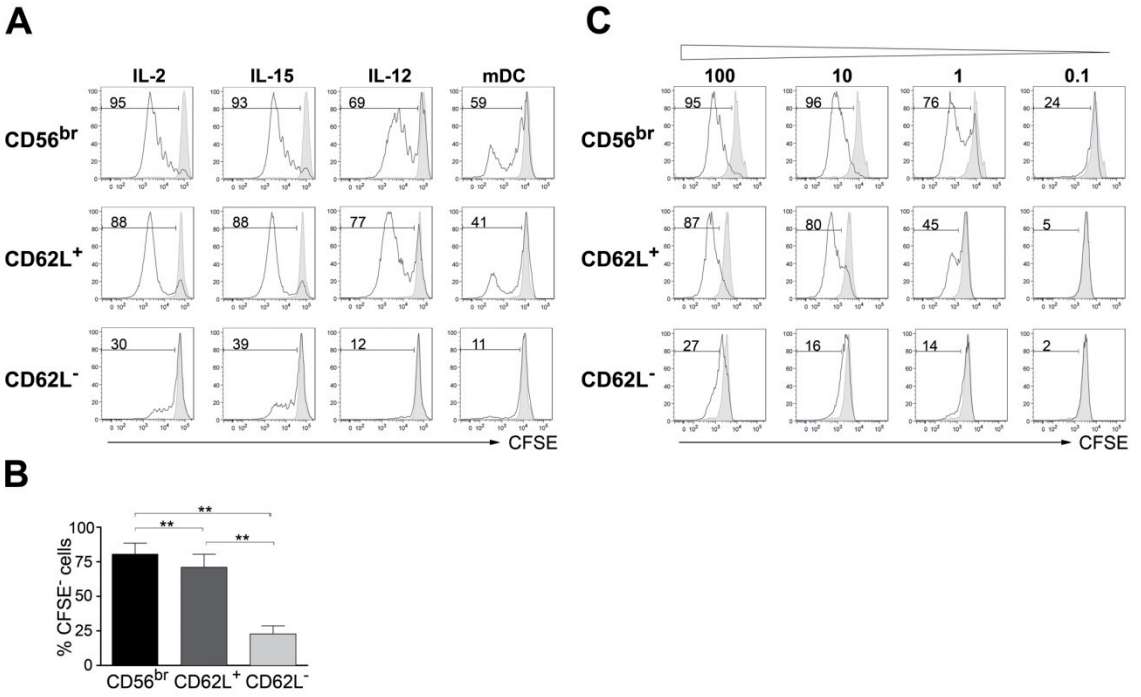


**Figure 10: CD56$^{bright}$ and CD56$^{dim}$ CD62L$^{+}$ NK cells display similar proliferative capacity *in vitro***

Analysis of in vitro proliferation of NK cell subsets was measured by CFSE labelling after different stimuli. (A) One representative experiment out of ten is shown. Stimulated (open histograms) and unstimulated (gray-filled histograms) cells as well as the percentage of proliferating cells are depicted for each condition. (B) Mean percentage ± SEM of proliferating cells after high dose of IL-2 stimulation of ten independent experiments is shown. **: $p< 0.01$ was calculated by Wilcoxon test. (C) In vitro proliferation of NK cell subsets was measured after decreasing concentrations of IL-2 (100ng/ml to 1ng/ml); one representative experiment out of four is shown.

To determine whether the ability to proliferate *in vitro* actually mirrors what happens *in vivo*, PBMCs derived from healthy donors were stained directly *ex vivo* for Ki67, which is expressed in $G_1$, $G_2$, S and M phases, but not in $G_0$ phase of the cell cycle (79, 80), and gated on T cells, total NK cells or the indicated NK cell subsets. In Figure 11 A, left panel, a representative Ki67 staining before vaccination in CD56$^{bright}$, CD56$^{dim}$ CD62L$^{+}$ and CD62L$^{-}$ NK cells is shown, while the right panel summarizes ex vivo expression of Ki67 in NK cell subsets of 22 donors.

These data clearly demonstrate that a consistent number of NK cells was undergoing homeostatic proliferation *in vivo* or has been recently activated due to an ongoing immune response and that within $CD56^{bright}$ and $CD56^{dim}$ $CD62L^{+}$ NK cells more in vivo proliferating cells can be detected compared to $CD62L^{-}$ ones. To investigate the proliferative response of NK cells in the course of a defined immune response, healthy donors were vaccinated with the live yellow fever virus (YFV)-17D. Immunization with YFV-17D vaccine results in a self-limiting acute viral infection with viral replication and therefore represents an ideal setting to assess NK cell proliferation during an immune response. Comparative analysis of Ki67 expression directly before and after YFV-17D vaccination revealed that the frequency of proliferating NK cells was significantly increased at day 7 compared to day 0 (Figure 11B, left) and returned to basal levels by day 28 (Figure 11B, middle). This data clearly indicates that a consistent proportion of NK cells were proliferating *in vivo* in response to viral infection. Importantly, cells which were proliferating at day 7 after YFV-17D vaccination were again comprised mostly within $CD56^{bright}$ and $CD56^{dim}$ $CD62L^{+}$ NK cell subsets (Figure 11B, right), demonstrating that the high proliferative ability displayed *in vitro* by these two subsets actually reflects their *in vivo* behaviour during a viral infection.

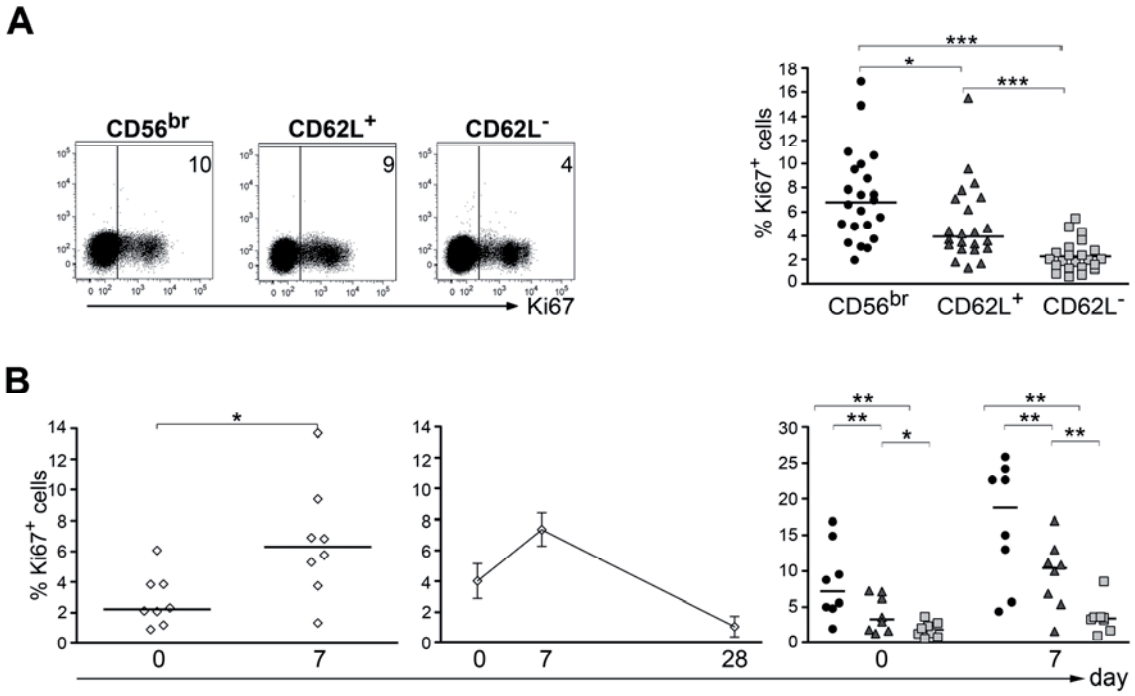


**Figure 11: CD56$^{bright}$ and CD56$^{dim}$ CD62L$^{+}$ NK cells display similar proliferative capacity *in vivo* after Yellow Fever Virus vaccination**

Analysis of *in vivo* proliferation of NK cell subsets was measured after ex vivo Ki67 staining of PBMC derived from healthy donors. (A) One representative donor (left panel) and mean percentage of Ki67$^{+}$ cells ± SEM of 22 donors analysed (right panel) is shown. (B) Ki67 expression was analysed directly before (day 0), or after YFV vaccination (day 7 and 28) gating on total CD3$^{-}$ CD56$^{+}$ NK cells (left and middle) or on CD56$^{bright}$ (black circles), CD56$^{dim}$ CD62L$^{+}$ (dark grey triangles) and CD56$^{dim}$ CD62L$^{-}$ (light grey squares) (right). Percentage of Ki67$^{+}$ cells of 8 donors and median of all values (left and right graphs) or mean percentage ± SEM of Ki67$^{+}$ cells of 3 donors (middle) are depicted. * $p<0.05$; ** $p<0.01$; *** $p< 0.0001$ as calculated by Wilcoxon test

Overall, these data show that CD56$^{bright}$ and CD56$^{dim}$ CD62L$^{+}$ NK cells have high proliferative ability both *in vitro* after stimulation with cytokines or dendritic cells and *in vivo* during an ongoing viral infection.

### *4.3.2 Differences in phosphorylation levels of STAT5 partially account for the higher responsiveness of CD56$^{dim}$ CD62L$^{+}$ cells to IL-2 and IL-15 stimulation*

The ability of CD56$^{bright}$ NK cells to efficiently respond to cytokine stimulation has been associated to the expression of the high affinity IL-2 receptor alpha chain, CD25 (22), and to higher phosphorylation levels of transcription factors such as ERK after cytokine stimulation (81). Since, CD56$^{dim}$ CD62L$^{+}$ cells show similar proliferative ability to CD56$^{bright}$ NK cells (Figure 10 and 11), it was analysed, whether differences in cytokine receptor expression could account for the superior proliferative ability of CD56$^{dim}$ CD62L$^{+}$ as compared to CD62L$^{-}$ NK cells. Yet, with the exception of the IL-2Rα chain, which was exclusively expressed on CD56$^{bright}$ cells as previously shown (22), CD56$^{bright}$ and both CD56$^{dim}$ subsets displayed similar amounts of the receptor subunits, analysed (Figure 12 A). Moreover, CD25 could still be up regulated between day 1 and 5 on CD56$^{dim}$ CD62L$^{+}$ as well as on CD62L$^{-}$ NK cells (data not shown). Therefore, while expression of CD25 on resting CD56$^{bright}$ cells might at least partially explain their higher proliferative ability, differences in cytokine receptor expression could not account for the distinct proliferative behaviour of CD56$^{dim}$ CD62L$^{+}$ or CD62L$^{-}$ cells. For that reason, phosphorylation of the STAT5, a transcription factor that becomes phosphorylated after activation of the common γ–chain receptor, was analysed. In line with that what has been shown for ERK (81), IL-2 (Figure 12 B) or IL-15 (Figure 12 B and C) stimulation resulted in high STAT5 phosphorylation in CD56$^{bright}$ cells. Notably, despite no major differences in cytokine receptor expression between the two CD56$^{dim}$ subsets, CD62L$^{+}$ displayed intermediate levels of p-STAT5 between CD56$^{bright}$ and CD62L$^{-}$ cells both after IL-2 (Figure 12 B) and IL-15 stimulation (Figure 12 B and C). These data imply that differences in phosphorylation intensity of STAT5 might at least partially account for the better responsiveness of CD56$^{dim}$ CD62L$^{+}$ NK cells to IL-2 and IL-15 stimulation.

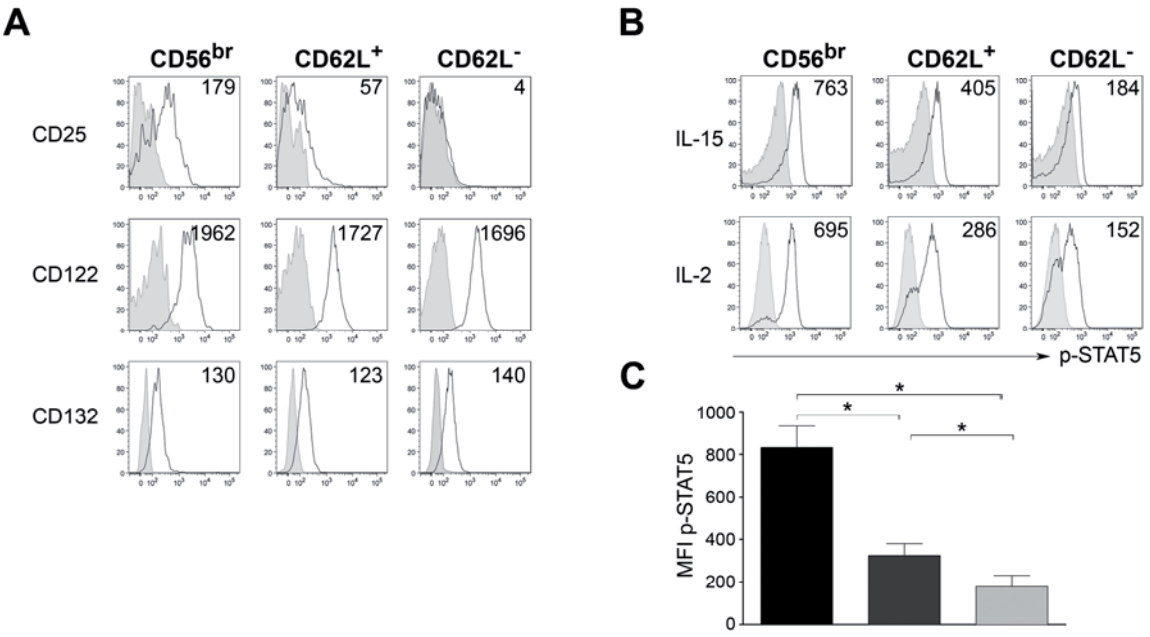


**Figure 12: Differences in Phosphorylation levels of STAT5 account for the higher responsiveness to IL-2 and IL-15 stimulation**

(A) Ex vivo staining of CD25 (IL-2Rα), CD122 (IL-15/IL-2Rβ chain) and CD132 (common γ chain) (open histogram) and corresponding isotype control staining (gray-filled histogram) on $CD56^{bright}$, $CD56^{dim}$ $CD62L^{+}$ and $CD56^{dim}$ $CD62L^{-}$ NK cells, MFI of specific staining minus MFI of isotype staining of one representative donor out of three is depicted. (B and C) Phosphorylation levels of STAT5 after stimulation of sorted $CD56^{bright}$, $CD56^{dim}$ $CD62L^{+}$ or $CD62L^{-}$ NK cells for 15 minutes with IL-15 (B, C) or IL-2 (B), one representative experiment out of six for IL-15 and out of 4 for IL-2 stimulation is shown. MFI of stimulated (black histogram) minus MFI of unstimulated control (filled grey histogramm) is depicted. (C) Mean p-STAT5 MFI ± SEM after stimulation in the presence of IL-15 of six independent experiment is shown, * $p<0.05$ as calculated by Wilcoxon test.

### 4.3.3 *CD56$^{dim}$ CD62L$^{+}$ expression identifies NK cells with high ability to produce IFN-γ after stimulation with cytokines or dendritic cells*

In addition to their high proliferative capacity, CD56$^{bright}$ NK cells are characterized by the higher ability to produce IFN-γ as compared to total CD56$^{dim}$ NK cells after stimulation with cytokines or dendritic cells (19, 30, 70). Since with respect to proliferation, CD56$^{dim}$ CD62L$^{+}$ behaves more similar to CD56$^{bright}$ than to the rest of the CD56$^{dim}$ (Figure 10- 12), IFN-γ production in both CD56$^{dim}$ subsets compared to CD56$^{bright}$ cells was analysed. As shown in Figure 13 A and B (left), CD56$^{bright}$ and CD56$^{dim}$ CD62L$^{+}$ NK cells contained similar percentages of IFN-γ secreting cells after stimulation with IL-12 and IL-18 or after co culture with PB-derived myeloid dendritic cells (Figure 13 A). Conversely, only a minority of CD62L$^{-}$ NK cells was able to produce IFN-γ under the same conditions. In addition, the amount of IFN-γ expression per cell as measured by mean fluorescence intensity (MFI) was significantly higher in CD56$^{bright}$ and in CD56$^{dim}$ CD62L$^{+}$ cells compared to CD62L$^{-}$ NK cells (Figure 13 B, right), thus showing that the first two subsets are competent IFN-γ producers. These findings indicate that also CD56$^{dim}$ CD62L$^{+}$ NK cells might contribute to an immune response with high amounts of IFN-γ after DC or cytokine stimulation and therefore could play an immune-regulatory role similar to what has been shown for CD56$^{bright}$ cells.

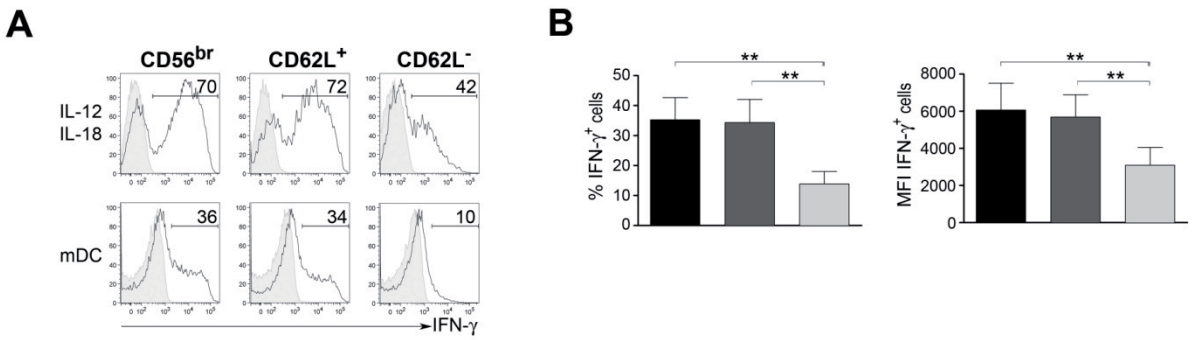


**Figure 13**: **IFN-γ production by NK cell subsets after cytokine stimulation**

IFN-γ production of CD56$^{bright}$, CD56$^{dim}$ CD62L$^{+}$ and CD62L$^{-}$ NK cells after stimulation with IL12+18 or with PB-derived myeloid DC activated with LPS and R848, (A) one representative experiment out of nine is shown. Stimulated (open histograms) and unstimulated (gray-filled histograms) as well as percentage of IFN-γ producing cells are depicted for each condition. (B) Mean percentage (left) and Mean MFI (right) of IFN-γ expressing cells ± SEM of nine independent experiments is shown, * $p<0.05$; ** $p<0.01$, as calculated by paired students t-test

### *4.3.4 Analysis of the impact of CD62L expression versus KIR, NKG2A or CD27 on proliferation and IFN-γ production*

CD62L expression clearly correlates with proliferation and IFN-γ production after cytokine or DC stimulation. However, apart from CD62L also KIR, NKG2A and CD27 have been shown to be differentially expressed within the $CD56^{dim}$ NK cell subset (65, 66) and have been associated to distinct maturation status and different functional properties of NK cells in humans and mice (37-39, 65, 66, 82). Yet, it has been previously demonstrated that $KIR^+$ NK cells proliferate less than $KIR^-$ NK cells (Figure5) and that $KIR^-$ $NKG2A^-$ NK cells proliferate less than $KIR^-$ $NKG2A^+$ ones (78). Moreover, it was shown that NK cells expressing self-MHC specific KIR or NKG2A produce more IFN-γ than NK cells lacking such receptors even after cytokine stimulation (39, 78). These results imply that expression of KIR or NKG2A can influence NK cell proliferative ability as well as their capacity to produce IFN-γ. In addition, it has been reported that $CD27^+$ NK cells have a higher proliferative ability than $CD27^-$ ones (66). In order to interpret all these results, we first analysed coexpression of CD62L with NKG2A, KIR and CD27 within NK cells. As shown in Figure 14 A (left), while all $CD56^{bright}$ NK cells are $CD62L^+$, $KIR^-$, $NKG2A^+$ and $CD27^{dim}$, the majority of $CD56^{dim}$ NK cells are $CD62L^-$, $KIR^+$, $NKG2A^-$, and $CD27^-$ with smaller proportion of cells expressing these markers. Analysis of coexpression of CD62L with NKG2A, KIR and CD27 in $CD56^{dim}$ NK cells (Figure 14 A, right, and B) revealed that although CD62L and KIR expression was not mutually exclusive, CD62L was present on a higher proportion of $KIR^-$ than $KIR^+$ NK cells among $CD56^{dim}$ NK cells in all donors analysed. Moreover, CD62L expression positively correlated with NKG2A and CD27 expression (Figure 14 A and B).

Thus, within the $CD56^{dim}$ NK cells, a subset of more immature cells exist that coexpresses CD62L and NKG2A, is negative for KIR and enriched in $CD27^+$ cells, similar to the phenotype of $CD56^{bright}$ cells.

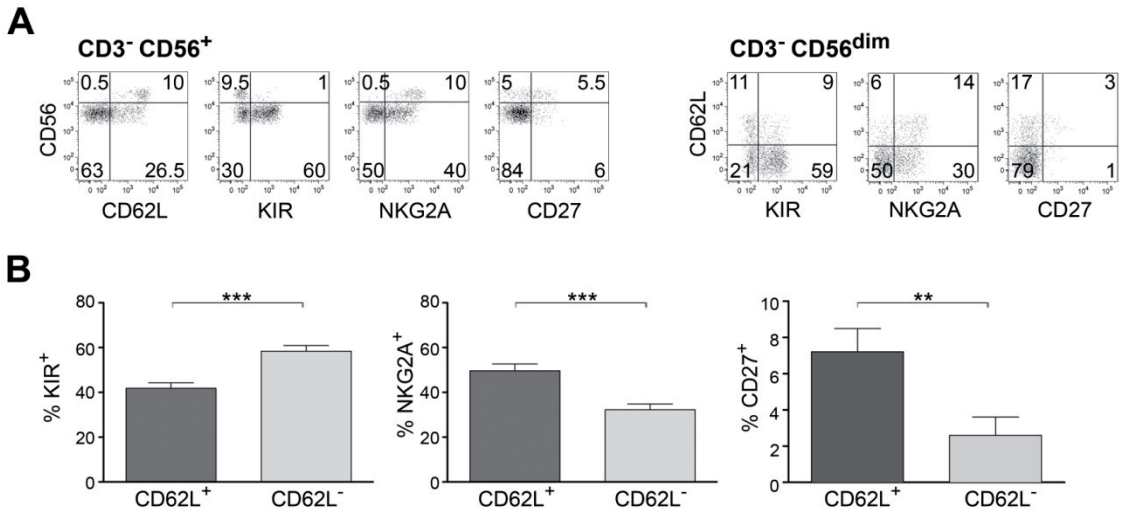


**Figure 14: Expression and Correlation of markers associated with NK cell maturation**

(A) FACS analysis of PBMCs gated on $CD3^-$ $CD56^+$ NK cells (left) or on $CD3^-$ $CD56^{dim}$ NK cells (right), one representative donor out of eleven is shown. (B) Mean percentages ± SEM of $KIR^+$, $NKG2A^+$ or $CD27^+$ cells within $CD56^{dim}$ $CD62L^+$ and $CD56^{dim}$ $CD62L^-$ of 26 (KIR), 22 (NKG2A) and 10 (CD27) different healthy donors are shown. ** $p<0.01$; *** $p< 0.0001$ as calculated by Wilcoxon test

Because of the positive correlation between CD62L and CD27 or NKG2A and the negative correlation between CD62L and KIR expression, it was tested which of these markers were directly associated to the ability to proliferate *in vivo* and to efficiently produce IFN-γ after cytokine stimulation. While *in vivo* proliferating cells were preferentially found within $CD56^{bright}$ and $CD56^{dim}$ $CD62L^+$ subsets (Figure 11 A), no significant correlation of Ki67 expression with CD27, KIR or NKG2A could be detected (Figure 15 A). These data suggest that CD62L and not KIR, NKG2A or CD27 expression identifies NK cells, which proliferate more extensively *in vivo*. Next, it was evaluated whether the capacity to produce IFN-γ after stimulation with cytokines would also correlate exclusively to CD62L expression, or rather to self-MHC class I specific inhibitory receptors such as KIR or NKG2A. Therefore, $CD56^{dim}$ NK cells derived from the PB of HLA-Bw4 individuals have been sorted into $CD62L^+$ $NKG2A^+$, $CD62L^+$ $NKG2A^-$, $CD62L^-$ $NKG2A^+$ and $CD62L^-$ $NKG2A^-$ subsets, stimulated with IL-12 plus IL-18 and afterwards stained for different KIR. IFN-γ expression was analysed in all sorted subsets after gating on "competent" cells that were single positive for the self-MHC class I (HLA-Bw4)-specific inhibitory receptor KIR3DL1 or on "hyporesponsive" cells negative for all KIRs.

Similar to proliferative ability, significantly more IFN-γ producing cells could be detected within CD56$^{dim}$ CD62L$^{+}$ than within CD62L$^{-}$ cells independently of KIR or NKG2A expression (Figure 15 B), thus clearly demonstrating that IFN-γ production in response to cytokines is not influenced by the presence of self MHC class I specific inhibitory receptors or NKG2A but rather correlates to CD62L expression. Once the possible impact of KIR and NKG2A was ruled out, the impact of CD27 expression was investigated. Again, IFN-γ secreting cells were significantly more enriched within the CD56$^{dim}$ CD62L$^{+}$ subset as compared to the CD62L$^{-}$ one, both in CD27$^{+}$ as well as in CD27$^{-}$ cells. Remarkably, the CD56$^{dim}$ CD27$^{+}$ CD62L$^{+}$ subset comprises more cells able to produce IFN-γ than the CD27$^{-}$ CD62L$^{+}$ one, demonstrating that CD56$^{dim}$ CD27$^{+}$ CD62L$^{+}$ cells, although representing a very minor population of NK cells (1.8 ± 0.3%), are the most potent IFN-γ producers within CD56$^{dim}$ NK cells (Figure 15 C).

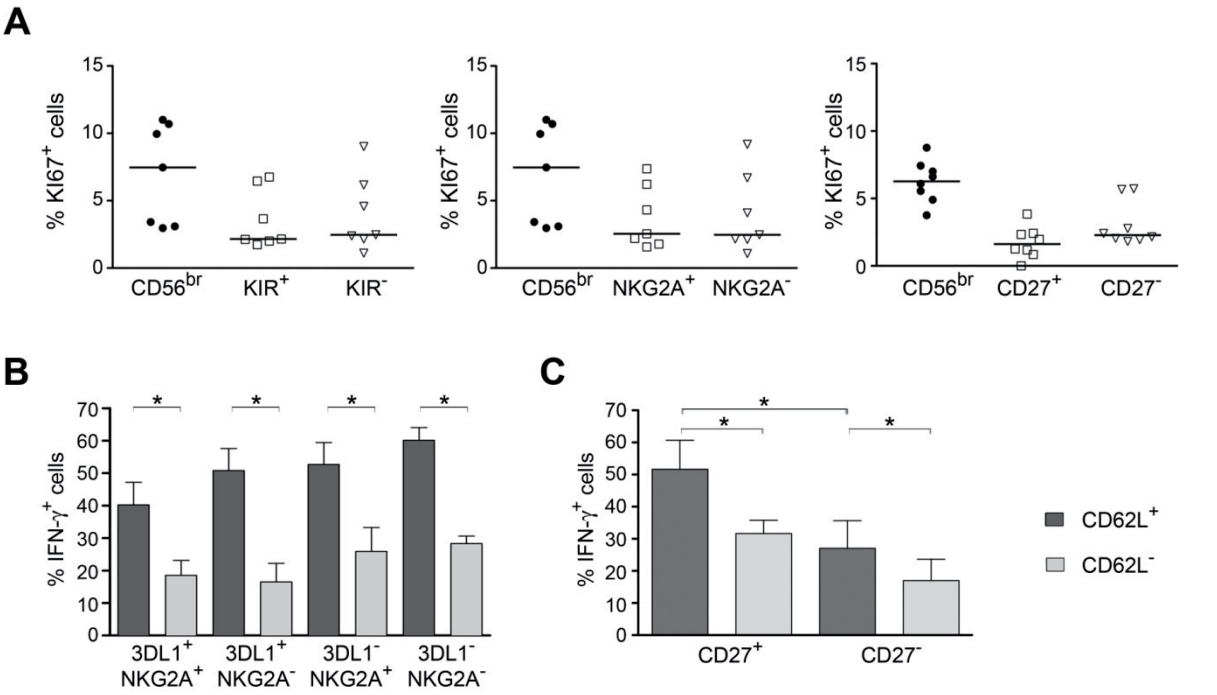


**Figure 15: Correlation of CD62L, CD27, KIR or NKG2A expression with NK cell proliferation and IFN-γ production**

(A) Ki67 expression in PB-NK cell subsets after staining for CD62L, 2+3D KIR (indicated as KIR), NKG2A and CD27 and gating on CD56$^{bright}$ or the indicated CD56$^{dim}$ subsets, 7 (KIR and NKG2A) or 8 (CD27) donors plus corresponding medians are shown. (B) CD56$^{dim}$ NK cells derived from HLA-Bw4 donors were sorted for NKG2A$^{-}$ CD62L$^{+}$ and NKG2A$^{-}$ CD62L$^{-}$ cells and stimulated with IL-12+IL-18. After additionally staining for 2D KIR and KIR3DL1 (indicated as 3DL1), IFNγ expression was analysed within competent 2D KIR$^{-}$ KIR3DL1$^{+}$ or hyporesponsive 2D KIR$^{-}$ KIR3DL1$^{-}$ cells.

Mean ± SEM of six independent experiments is shown. (C) CD56$^{dim}$ NK cells were sorted for CD27 and CD62L expression and IFN-γ was analysed after stimulation with IL-12+18. Mean values ± SEM of six independent experiments are shown. * $p<0.05$ as calculated by Wilcoxon test.

### *4.3.5 Both, CD62L$^+$ and CD62L$^-$ CD56$^{dim}$ are able to respond to activating receptor stimulation*

NK cells cannot only produce IFN-γ after cytokine stimulation but also upon engagement of activating receptors by stimulatory ligands expressed on target cells. It has been shown that simultaneous triggering of multiple activating receptors results in cytokine production and cytotoxicity by human resting NK cells (83). Therefore, IFN-γ and TNF expression after cross-linking of multiple activating receptors was analysed in CD56$^{bright}$, CD56$^{dim}$ CD62L$^+$ and CD62L$^-$ cells. Surprisingly, although CD56$^{bright}$ cells are generally considered the cytokine producers, they did not express significant amounts of either IFN-γ or TNF after activating receptor stimulation (Figure 16 A). In contrast, both CD56$^{dim}$ cell subsets, CD56$^{dim}$ CD62L$^+$ and CD62L$^-$ cells, were proficient IFN-γ and TNF producers after activating receptor stimulation (Figure 16 A). These data show for the first time that during differentiation from CD56$^{bright}$ to CD56$^{dim}$, NK cells lose the ability to produce cytokines after cytokine stimulation but acquire the capacity to express it after activating receptor stimulation. Comparative analysis of cytotoxicity towards MHC class I negative tumour target cells revealed that CD56$^{dim}$ CD62L$^+$ and CD62L$^-$ NK cells were both able to kill, while CD56$^{bright}$ cells displayed much lower cytotoxic ability (Figure 16 B), in line with previous observations (33). Indeed, although CD56$^{dim}$ CD62L$^+$ NK cells display many functional and phenotypic features in common with CD56$^{bright}$, cytotoxic ability (Figure 16 B) as well as *ex vivo* expression of perforin, granzymeA and granzymeB (Figure 16 C) was comparably high in CD56$^{dim}$ CD62L$^+$ and in CD62L$^-$ cells. Thus, these data demonstrate that CD56$^{dim}$ CD62L$^+$ NK cells are not only able to extensively proliferate and to produce IFN-γ after cytokine stimulation similar to what has been shown for CD56$^{bright}$ NK cells, but are also capable of killing and producing cytokines after activating receptor stimulation as the rest of CD56$^{dim}$ NK cells, therefore representing a unique subset of polyfunctional NK cells.

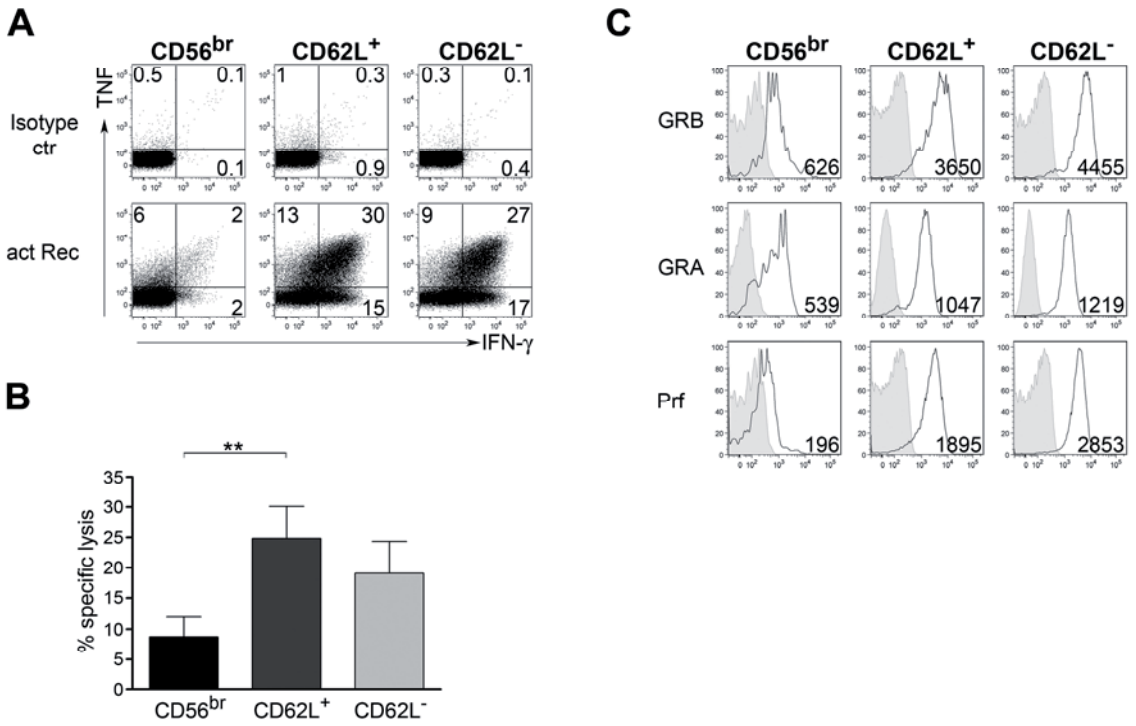


**Figure 16: Analysis of cytotoxicity and cytokine production after activating receptor stimulation**

**(A)** Analysis of intracellular IFN-γ and TNF expression in $CD56^{dim}$ $CD62L^{+}$ and $CD62L^{-}$ NK cells after stimulation with a combination of plate-bound mAb against NKp30, NKp46, NKG2D, 2B4 and CD2 or isotype control mAb; one representative experiment out of three is shown. **(B)** Analysis of cytotoxicity of $CD56^{bright}$, $CD56^{dim}$ $CD62L^{+}$ and $CD62L^{-}$ NK cells after K562 stimulation; mean ± SEM of nine independent experiments is shown. **(C)** *Ex vivo* expression of granzymeA (GRA), granzymeB (GRB) and perforin (Prf) (open histogram) and corresponding isotype control staining (gray-filled histogram) in $CD56^{bright}$, $CD56^{dim}$ $CD62L^{+}$ and $CD56^{dim}$ $CD62L^{-}$ NK cells; one representative donor out of three is shown.

Since cytotoxic competence has been demonstrated to correlate with the presence of self-MHC specific inhibitory receptors, it was investigated more in detail whether CD62L expression can influence NK cell competence within $KIR3DL1^{+}$ cells (competent) or $KIR^{-}$ $NKG2A^{-}$ (hyporesponsive) cells in HLA-Bw4 donors. As previously shown (39, 40), expression of self-MHC specific KIRs such as KIR3DL1 results in the most striking correlation with cytotoxic competence (Figure 17 A). However, expression of CD62L confers a slightly higher cytotoxic potential to both competent ($KIR3DL1^{+}$) as well as hyporesponsive ($KIR^{-}$ $NKG2A^{-}$) NK cells, which could result from recent *in vivo* activation of $CD56^{dim}$ $CD62L^{+}$ NK cells by cytokines.

The degree of the effect associated with CD62L expression was comparable or even higher than the one associated with NKG2A (Figure 17 B). Interestingly, within hyporesponsive ($KIR^-$ $NKG2A^-$) NK cells, those, which are potentially autoreactive, are almost exclusively included within the $CD56^{dim}$ $CD62L^+$ subset (Figure 17 B). In the first two parts of this thesis it was shown that $CD56^{dim}$ $KIR^-$ (hyporesponsive) NK cells can up-regulate KIR after cytokine stimulation (Figure 5) and that only those cells which *de novo* express self MHC specific KIR are licensed to kill (Figure 9). We therefore asked whether cytokine stimulation of $CD56^{dim}$ $KIR^-$ $CD62L^+$ or $KIR^-$ $CD62L^-$ NK cells would result in comparable KIR *de novo* expression. As shown in Figure 17 C, KIR expression could be induced on both $KIR^-$ $CD62L^+$ as well as $KIR^-$ $CD62L^-$ NK cells. However, a consistently higher proportion of $KIR^+$ cells could be detected among $CD56^{dim}$ $CD62L^+$ compared to $CD62L^-$ NK cells, suggesting that cells endowed with strong proliferative ability might have a higher chance to be licensed.

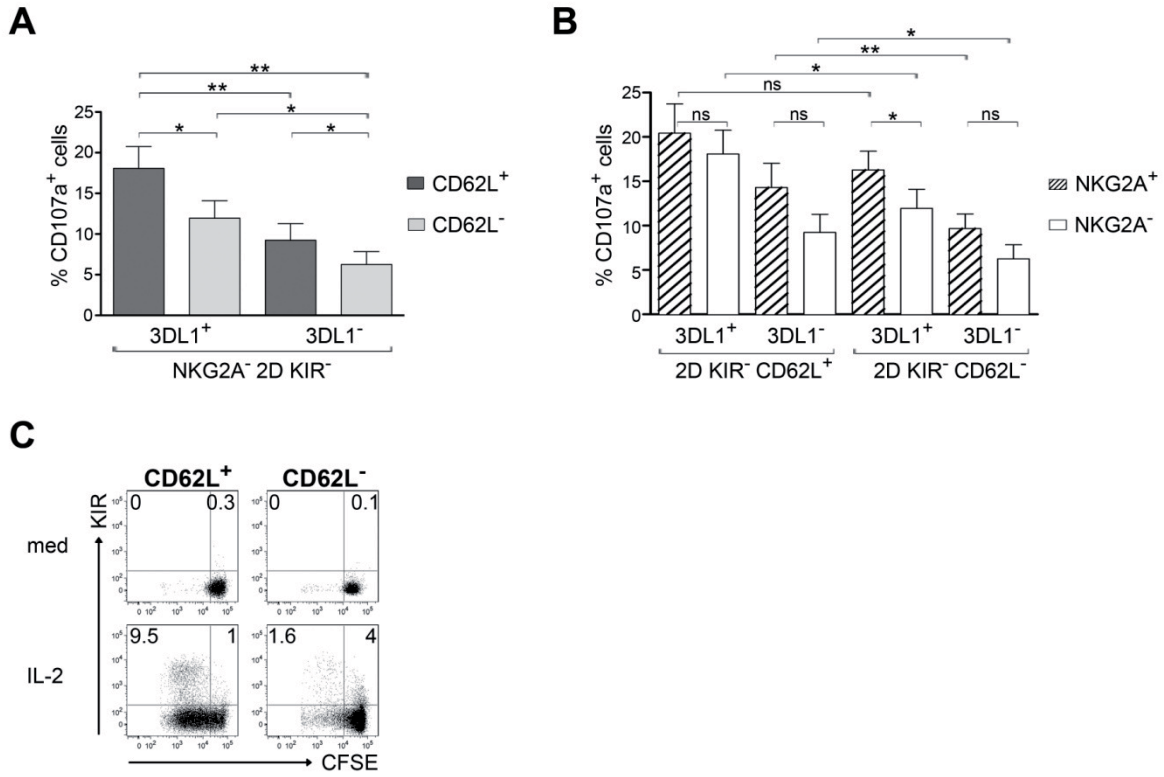


**Figure 17: Correlation of CD62L, KIR and NKG2A with cytotoxic competence**

**(A)** $CD56^{dim}$ NK cells derived from HLA-Bw4 donors were sorted for $NKG2A^-$ $CD62L^+$ and $NKG2A^-$ $CD62L^-$ cells and stimulated for 6h with K562. CD107a expression was analysed within competent $KIR3DL1^+$ 2D $KIR^-$ $NKG2A^-$ and hypo responsive $KIR3DL1^-$ 2D $KIR^-$ $NKG2A^-$ NK cell subsets after staining for KIR3DL1 (indicated as 3DL1) and 2D KIRs and gating on the indicated subsets: $CD56^{dim}$ $CD62L^+$ cells are depicted in dark grey and $CD62L^-$ ones in light grey. Mean ± SEM of eight inde-

pendent experiments is shown. * p<0.05; ** p<0.01; *** p< 0.0001 as calculated by Wilcoxon test, **(B)** CD56$^{dim}$ NK cells derived from HLA-Bw4 donors were sorted for CD62L and NKG2A expression and stimulated for 6h with K562. CD107a expression was analysed within 2D KIR$^-$ KIR3DL1$^+$ or 2D KIR$^-$ KIR3DL1$^-$ cells. Mean values ± SEM of eight independent experiments are shown. * p<0.05; ** p<0.01; ns indicates p>0.05, as calculated by Wilcoxon test **(C)** Sorted CD56$^{bright}$ 2+3D KIR$^-$, CD56$^{dim}$ 2+3D KIR$^-$ CD62L$^+$ or CD56$^{dim}$ 2+3D KIR$^-$ CD62L$^-$ NK cells were CFDA labelled, cultivated in the presence of IL-2 for 5 days and analysed for 2+3D KIR expression. Percentage of KIR induction is shown. One representative experiment out of three is depicted.

### *4.3.6 CD56$^{dim}$ CD62L$^+$ KIR$^-$ NK cells have intermediate telomere length between CD56$^{bright}$ and CD56$^{dim}$ CD62L$^-$ KIR$^+$ NK cells*

Since CD56$^{dim}$ CD62L$^+$ NK cells have a more immature phenotype, i.e. being KIR$^-$ NKG2A$^+$ CD27$^+$ similar to CD56$^{bright}$ cells, and as they combine the ability to respond to cytokines with the ability to respond to activating receptor stimulation, especially when self-MHC specific KIR are coexpressed, CD56$^{dim}$ CD62L$^+$ NK cells could represent an intermediate step of NK cell differentiation between CD56$^{bright}$ and CD56$^{dim}$ CD62L$^-$ cells. In order to test this hypothesis, telomere length in different NK cell subsets were measured. Comparative analysis of CD62L$^+$ and CD62L$^-$ or of KIR$^+$ and KIR$^-$ among CD56$^{dim}$ NK cells revealed no significant difference in telomere length (data not shown). However, telomere length analysis of cells, which have been sorted according to CD62L and KIR together, showed that CD56$^{dim}$ CD62L$^+$ KIR$^-$ displayed intermediate telomere length between CD56$^{bright}$ and CD56$^{dim}$ CD62L$^-$ KIR$^+$ NK cells (Figure 18 A). These data suggest that within the CD62L$^-$ subset, cells, which have acquired KIR expression, displayed the longest proliferative history and therefore differences in telomere length became detectable. Since loss of CD62L and acquisition of KIR expression appear to be uncoordinated events, and are probably induced by different stimuli, CD62L$^+$ KIR$^+$ or CD62L$^-$ KIR$^-$ cells also exist but differences in their telomere length may not be substantial. It has been demonstrated that NK cells obtained from elderly people display increased frequencies of CD56$^{dim}$ cells and decreased frequencies of CD56$^{bright}$ cells (84), suggesting that terminally differentiated NK cells accumulate with ageing, similar to what has been shown for T cells (85). When we analysed the percentage of CD56$^{bright}$, CD56$^{dim}$ CD62L$^+$ and CD62L$^-$ NK cells in correlation to age, we found that frequencies of CD56$^{bright}$ as well as of CD56$^{dim}$ CD62L$^+$ NK cells progressively decreased with ageing, while CD62L$^-$ cells increased (Figure 18 B).

Altogether these data suggest that CD56$^{dim}$ CD62L$^{+}$ NK cells are enriched in cells displaying an intermediate step of maturation between CD56$^{bright}$ and CD56$^{dim}$ CD62L$^{-}$ NK cells.

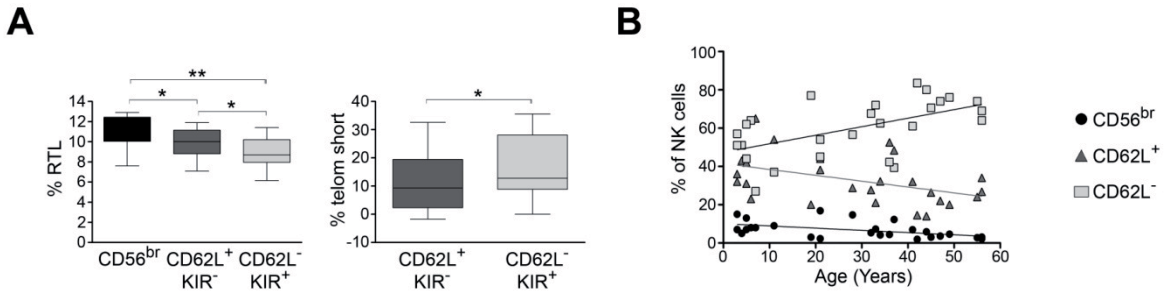


**Figure 18: CD56$^{dim}$ CD62L$^{+}$ NK cells have intermediate telomere length and decrease with ageing**

**(A)** Analysis of telomere length of CD56$^{bright}$ (black), CD56$^{dim}$ CD62L$^{+}$ KIR$^{-}$ (dark grey) and CD56$^{dim}$ CD62L$^{-}$ KIR$^{+}$ (light grey) NK cells, box plots with median and interquartile range of nine different donors are shown. * $p<0.05$; ** $p<0.01$ as calculated by paired students t test. **(B)** Analysis of percentage of CD56$^{bright}$ ($r = -0.56$, $p= 0.003$), CD56$^{dim}$ CD62L$^{+}$ ($r = -0.44$, $p=0.02$, and CD56$^{dim}$ CD62L$^{-}$ ($r = 0.56$, $p=0.003$) in correlation to age, Pearson correlation coefficient of 26 donors has been calculated.

### *4.3.7 CD56$^{dim}$ CD62L$^{+}$ NK cells have the potential to home to lymph nodes*

CD62L allows interaction with glycosylated L-selectin ligands on high endothelial venules and it is crucial for T cell and NK cell homing to lymph nodes (6, 86). However, it is unclear whether CD62L alone is sufficient to enter lymph nodes (87). CD56$^{bright}$ cells, which preferentially reside in lymph nodes, express not only CD62L but also the chemokine receptor CCR7 (8). Conversely, CD56$^{dim}$ NK cells are mostly CCR7$^{-}$ but express CXCR1, which enable them to migrate into inflamed tissues (8). In order to understand the migration potential of CD56$^{dim}$ CD62L$^{+}$ cells, the expression of CCR7 and CXCR1 in NK cell subsets isolated *ex vivo* or after stimulation was reanalysed. CCR7 was indeed present not only on CD56$^{bright}$ but also on a small fraction of CD56$^{dim}$ cells. In line with CCR7 expression, few CXCR1$^{-}$ cells do also exist within CD56$^{dim}$ cells.

Interestingly, almost all CD56$^{dim}$ cells that were CCR7$^{+}$ or CXCR1$^{-}$ were CD62L$^{+}$ and a consistent proportion of them coexpressed CD27 (Figure 19 A). While CCR7 and CXCR1 expression was mutually exclusive within CD56$^{bright}$ and CD56$^{dim}$ CD62L$^{-}$ cells, a minority of CD56$^{dim}$ CD62L$^{+}$ cells coexpressed CXCR1 (Figure 19 A). Short IL-18 stimulation is able to induce CCR7 expression in CD56$^{dim}$ cells (59). When CD56$^{dim}$ CD62L$^{+}$ and CD62L$^{-}$ cells were analysed for CCR7 expression after IL-18 stimulation, CCR7 was up-regulated by the majority of CD56$^{dim}$ CD62L$^{+}$ cells (Figure 19 B) but only by a minor fraction of CD62L$^{-}$ ones. Altogether, these data suggest that CD56$^{dim}$ CD62L$^{+}$ cells have the potential to home to lymph nodes similar to CD56$^{bright}$ cells.

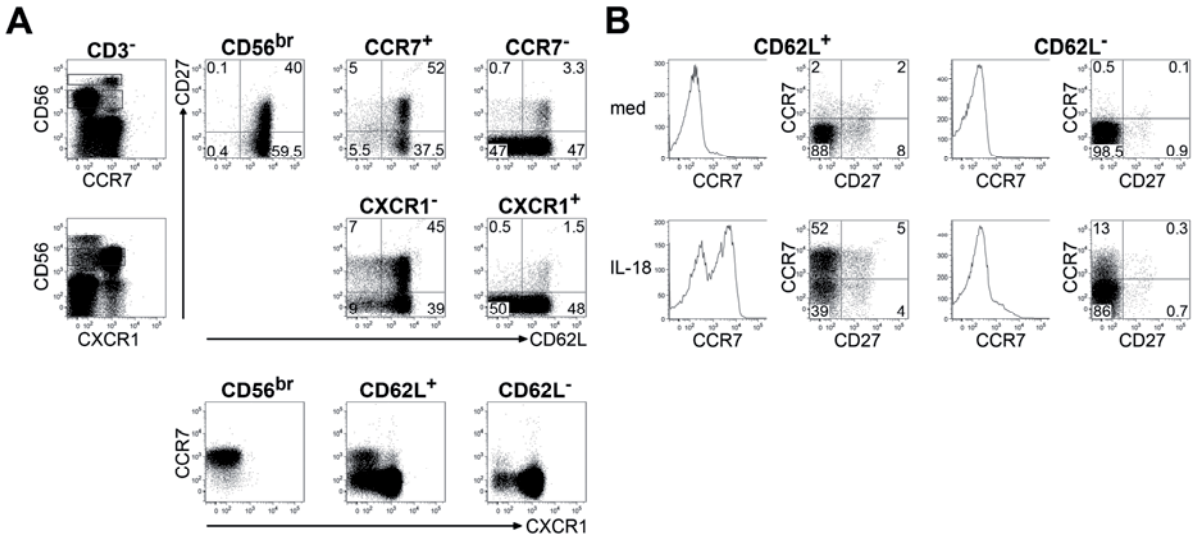


**Figure 19: Analysis of chemokine receptor expression *ex vivo* and after stimulation of NK cell subsets**

(A) *Ex vivo* analysis of CCR7, CXCR1, CD62L and CD27 coexpression after gating on the indicated subsets; one representative donor out of three is shown. (B) Modulation of CCR7 expression after stimulation of sorted CD56$^{bright}$, CD56$^{dim}$ CD62L$^{+}$ and CD56$^{dim}$ CD62L$^{-}$ NK cells for 36 h in medium or in the presence of IL-18

# 5 Discussion

In the frame of this thesis, three central questions have been explored.

First, the developmental relation between PB-CD56$^{bright}$ and CD56$^{dim}$ NK cells and potential sites of terminal NK cell maturation has been investigated. The data presented in the first part of this thesis provide new evidences strengthening the hypothesis that CD56$^{bright}$ may be precursors of CD56$^{dim}$ NK cells and that NK cell final maturation and acquisition of competence might occur in SLO during an inflammatory response.

Second, it was analysed whether cytokines can induce expression of self specific inhibitory receptors and if so whether that would lead to education of hyporesponsive NK cells to become competent and self tolerant. It could be demonstrated that cytokine induced expression of KIR leads to the generation of competent NK cells if the corresponding self-MHC molecule is present in the donor.

The aim of the third part of this thesis was to evaluate whether the expression of CD62L or other markers might be used to identify intermediate stages of NK cell maturation characterised by distinct functional properties and in line with that better define NK cell developmental history. It was shown that CD56$^{dim}$ CD62L$^{+}$ cells represent an intermediate stage of NK cell maturation, which after restimulation can accomplish multiple functional tasks, and further develop into terminally differentiated effectors. This subset is unique in the way that it is responsive to cytokine stimulation as well as to activating receptor stimulation, abilities which are otherwise mutually exclusive in the rest of the NK cell population.

## 5.1 CD56$^{bright}$ NK cells display longer telomeres and acquire features of CD56$^{dim}$ NK cells upon cytokine activation

Although the two main PB-human NK cell subsets, which are CD56$^{bright}$ and CD56$^{dim}$ NK cells, have been characterized regarding their functional properties, phenotype and tissue localisation, and despite progress in understanding NK cell specificity for target cells, less is known about stages of terminal NK cell maturation, expansion and site of differentiation, especially in the human system. In the first part of this thesis (chapter 3.1), the developmental relationship between CD56$^{bright}$ and CD56$^{dim}$ NK cells and the site of terminal human NK cell differentiation has been investigates more in detail. The data presented here show that after cytokine activation, in particular IL-2 and IL-15, CD56$^{bright}$ NK cells could acquire the signature of CD56$^{dim}$ NK cells, i.e. KIR$^{+}$ CD16$^{+}$ IL7R$\alpha^{-}$ c-kit$^{-}$ CXCR3$^{-}$ CCR7$^{-}$, CD62L$^{-}$, while CD56$^{dim}$ CD16$^{+}$ KIR$^{+}$ NK cells maintain their features of terminally differentiated cells. Within the molecules induced in CD56$^{bright}$ NK cells, KIRs are of great interest. In contrast to CD16 expression, which has been shown to be differentially modulated depending on the stimuli (Figure 4) or the experimental model used (58, 59), KIR expression remains stable as shown in NK cell clones under varying cell culture conditions and activation stimuli (88, 89). Clonal patterns of KIR expression are mainly epigenetically regulated and maintained through DNA methylation (88, 89). In line with that, so far KIR expression was induced *in vitro* only using methyltransferase inhibitors. However, as demonstrated in the present study, both CD56$^{bright}$ KIR$^{-}$ and CD56$^{dim}$ KIR$^{-}$ NK cells could express KIR on a subset of cells after cytokine stimulation. This result is in fact in contrast to a previous publication where it was claimed that no KIR or CD16 up-regulation was occurring on CD56$^{bright}$ NK cells after IL-2 stimulation (9). One possible explanation for this discrepancy might rely on the different experimental conditions (e.g. IL-2 concentration).

The evidence that KIR are molecules inducible on NK cells raises interest about the mechanisms by which this process can occur, since this understanding might help to clarify the mechanisms underlying NK cell tolerance to self. For long time it was claimed that each NK cell expresses at least one inhibitory receptor specific for self-MHC class I thereby ensuring tolerance towards self. However, recent studies have changed the view on how NK cell self-tolerance is achieved, showing that NK cells, which do not express inhibitory receptors recognizing self-MHC, do exist. Nonetheless, only NK cells expressing self-MHC specific inhibitory receptors are competent, while those lacking such receptors display an anergic phenotype (37-39, 41, 42). In this context, the finding that KIR can be induced by cytokines is of great interest because it suggests that NK cell self-tolerance might be a dynamic process probably related to cell differentiation: Cytokines produced during an inflammatory response by DC or T cells could induce NK cell differentiation and thereby generation of new competent NK cells. Along this line, it has been shown that also Ly49 molecules, the corresponding receptors in mice, can be modulated on NK cells after cytokine stimulation, suggesting a similar scenario in mice (90).

As shown in Figure 5, $KIR^+$ NK cells showed lower ability to proliferate in response to cytokines compared not only to $CD56^{bright}$ but also to $CD56^{dim}$ $KIR^-$. This surprising finding was not due to a mAb staining artefact (e.g. inhibition of proliferation via triggering of inhibitory receptors by anti-KIR mAb) since staining or not total $CD56^{dim}$ NK cells (mixture of $KIR^+$ and $KIR^-$ NK cells) with anti-KIR mAb combination used for the sorting procedure did not influence at all NK cell proliferation (data not shown). One possible explanation of the lower $CD56^{dim}$ $KIR^+$ NK cell proliferation might be due to KIR binding in cis or in trans to surface MHC class I molecules expressed on NK cells, which could result in inhibition of proliferation after stimulation, as it has been shown for cis-binding of Ly49 in mice (91). Nonetheless, since, also in this case, staining of KIR with mAb should influence KIR ability to bind MHC class I molecules and resulting in less inhibition, this is rather unlikely. Given all these observations, the hypothesis can be favoured that $CD56^{bright}$ and $CD56^{dim}$ NK cells do not belong to two distinct subsets, but rather that $CD56^{bright}$ represent an earlier stage of NK cell development and that KIR acquisition correlates with a terminal step of NK cell maturation, as it has been already speculated both for NK cells and $CD8^+$ $KIR^+$ T cells (92, 93).

As shown already for T cells, terminal differentiation towards an effector cell type goes along with the loss of proliferative capacity. Next, modulation of cytokine receptors, such as the stem cell factor receptor CD117 (c-kit) and CD127 (IL7Rα), that have been shown to be present not only on $CD56^{bright}$ but already on NK cell immature precursors in humans and in mice (55, 94) has been investigated. It could be shown that these receptors were also down regulated on $CD56^{bright}$ NK cells during cytokine-induced proliferation. The progressive loss of these receptors is also very suggestive of a differentiation process from an early to a more advanced stage at which NK cells do not require stem cell factor or IL-7 signalling any longer. Accordingly, mouse $Mac^{low}$ NK cells displaying an immature phenotype tend to express c-kit, which is then absent in mature $Mac^{hi}$ NK cells (94). $CD56^{bright}$ NK cells express lymph node homing receptors such as CD62L, CXCR3 and CCR7 and represent the majority of NK cells found in SLO. Down-regulation of these SLO homing molecules on $CD56^{bright}$ NK cells upon cytokine stimulation, as shown in Figure 3, is consistent with the hypothesis that these NK cells would leave SLO after their activation. *In vitro* differentiation of $CD56^{bright}$ NK cells towards a phenotype akin of $CD56^{dim}$ NK cells was often accompanied by extensive NK cell proliferation induced by cytokine stimulation. Thus, if $CD56^{bright}$ NK cells represented an earlier developmental step of NK cell differentiation, they should have undergone a lower number of proliferative events *in vivo*. To test this hypothesis, telomere length of $CD56^{bright}$, $CD56^{dim}$ an LN-NK cells in comparison to naive and memory T cells were evaluated, since in most normal somatic cells telomere length inversely correlates with proliferative history. $CD56^{bright}$ NK cells displayed longer telomeres than $CD56^{dim}$ NK cells, and telomere-shortening in $CD56^{dim}$ compared to $CD56^{bright}$ was similar to the one observed in memory T cells compared to naive T cells. In this regard, $CD56^{bright}$ exhibit the same characteristics as naïve T cells, i.e. longer telomeres compared to memory T cells, in according to previous reports (72). Although not providing the definitive proof that $CD56^{bright}$ are the precursors of $CD56^{dim}$ NK cells, these results definitely rule out the possibility that $CD56^{bright}$ are derived from $CD56^{dim}$ ones. Very recently, other studies have provided further evidences that $CD56^{bright}$ are the precursors of $CD56^{dim}$ NK cells. Indeed, it was shown that $CD56^{bright}$ NK cells acquire the phenotype of $CD56^{dim}$ cells after *in vitro* culture with IL-15 and murine stromal cells (78) or with human fibroblasts and that $CD56^{bright}$ NK cells have longer telomeres than $CD56^{dim}$ ones (95). Furthermore, a study in humanized $Rag2^{-/-}\gamma c^{-/-}$ reconstituted with human HPC supports an IL-15 dependent linear differentiation from immature $CD56^{bright}$ $CD16^{-}$ $KIR^{-}$ over $CD56^{dim}CD16^{+}$ $KIR^{-}$ to $CD56^{dim}$ $CD16^{+}$ $KIR^{+}$ NK cells (96). Altogether, the results obtained in this thesis together with other published data strongly support the concept,

that $CD56^{bright}$ NK cells are more immature and can give rise to $CD56^{dim}$ NK cells under the influence of cytokines.

To get an idea what could be actually the site of terminally differentiation of $CD56^{bright}$ into $CD56^{dim}$ NK cells, *ex vivo* analysis of human NK cells from different compartments were performed. This analysis revealed that non-reactive LNs contain almost exclusively $CD56^{bright}$ $KIR^{-}$ $CD16^{-}$ NK cells while a significant expression of KIR and CD16 on NK cells was present in highly inflamed LN and in the efferent lymph. These data suggest that $CD56^{bright}$ $KIR^{-}$ $CD16^{-}$ NK cells can acquire KIR and CD16 in inflamed LN and then circulate as $KIR^{+}$ $CD16^{+}$ NK cells via the efferent lymph in PB. The assumption that cytokines released during inflammation can mobilize NK cells from SLO to PB is also supported by previous studies reporting that recombinant IL-2 therapy for human cancer results in a striking increase of $CD56^{bright}$ $CD16^{+}$ NK cells in PB (24). $CD56^{bright}$ $KIR^{-}$ NK cells may re-circulate through SLO or reside there where they become activated by cytokines inducing proliferation and maturation into $KIR^{+}$ $CD16^{+}$ cytotoxic NK cells, and later leave LN in order to reach inflamed tissues. Although this hypothesis is very challenging, it cannot be excluded that the presence of $KIR^{+}$ $CD16^{+}$ NK cells in inflamed LN and in the efferent lymph might be due to selective migration of this subset into LN and not to $CD56^{bright}$ differentiation into $CD56^{dim}$ NK cells. Nonetheless, since it has been shown that NK cell recruitment into inflamed LN occurs via CD62L and CXCR3 (6), $KIR^{+}$ $CD16^{+}$ NK cells, which are preferentially $CD62L^{-}$ and $CXCR3^{-}$, should be less prone to migrate to this site.

There are other studies suggesting that LN may represent a key site for NK cell development (52, 55). In fact, it has been recently shown that four different developmental stages of human NK cell precursors are present in LN and that differentiation from these precursors to mature $CD56^{bright}$ NK cells can be mediated by cytokines and are supported by stromal cells (52, 55). However, from these previous observations it is not yet clear to what extent NK cell differentiation in SLO might account for the total mature NK cell compartment in the body, as most NK cells in human PB are $CD56^{dim}$. The data presented in this thesis suggest that also final maturation of $CD56^{bright}$ into $CD56^{dim}$ might occur in SLO, further supporting the hypothesis that $CD56^{dim}$ NK cells might correspond to the terminally differentiated stage of human NK cell development. Although reactive SLO might represent an important site of NK cell differentiation and maturation, such differentiation processes could also take place in other inflamed tissues, where both $CD56^{bright}$ and $CD56^{dim}$ NK cells can be found (97).

Whatever the case, it would be very interesting to investigate which cell type resident in the LN is crucial to induce NK cell proliferation and maturation. One interesting candidate is the dendritic cell (DC) since NK cells and DCs are colocalized in LN paracortex and medulla, and have been shown to interact with each other over extended times (98, 99). Since it was previously shown that both myeloid and plasmacytoid DCs can induce selective expansion of $CD56^{bright}$ NK cells (70, 99, 100), it would be important to determine whether DCs can induce not only $CD56^{bright}$ proliferation but also differentiation into $CD56^{dim}$ NK cells. To this aim, whether DCs can induce KIR and CD16 expression on proliferating $CD56^{bright}$ NK cells should be analysed. It is also not clear at which phase of an immune response NK cell final maturation may happen. Both DC- (IL-15 and to some extent IL-12) and T cell- (IL-2) derived cytokines can induce this differentiation step *in vitro*. If DC-derived cytokines were primarily involved in NK cell maturation *in vivo*, this process could take place in the very early phase of an innate immune response before T cell clonal expansion. However, considering the effect of IL-2 *in vitro* and the significant *in vivo* association between KIR/CD16 expression and paracortical/follicular hyperplasia where extensive lymphocyte proliferation occurs, it is conceivable that NK cell terminal differentiation could take place later on during an immune response, when proliferating naïve T cells start to produce high amounts of IL-2.

In conclusion, the data presented in the first part of this thesis provide new evidences supporting the hypothesis that $CD56^{bright}$ may give raise to $CD56^{dim}$ NK cells and a scenario in which NK cell final maturation and acquisition of competence might occur in SLO during an inflammatory response can be hypothesized.

## 5.2 Cytokines induce stable expression of inhibitory receptors on hyporesponsive CD56$^{dim}$ KIR$^{-}$ NKG2A$^{-}$ NK cells

In the first part of this thesis it could be demonstrated that cytokines such as IL-2, IL-15 and to a lower extend IL-12 can induce *de novo* expression of KIR in PB- or LN-derived CD56$^{bright}$ KIR$^{-}$ or CD56$^{dim}$ KIR$^{-}$ NK cells. Additionally, it was shown that KIR$^{+}$ NK cells are detectable only in highly reactive lymph node, characterized by lymphocyte expansion and cytokine production. Since expression of self-MHC class I specific inhibitory receptors such as KIR and NKG2A is essential to ensure self-tolerance and to render a NK cell functional competent, it was investigated whether cytokine induced expression of self-specific inhibitory receptors would be enough to educate KIR$^{-}$ NKG2A$^{-}$ hyporesponsive NK cell. The data presented in the second part of this thesis (chapter 3.2) provide the first evidence that acquisition of KIR expression after cytokine stimulation leads to education of hyporesponsive NK cells. In fact, it was clearly demonstrated that cytokines are able to induce stable KIR expression on a subset of hyporesponsive KIR$^{-}$ NKG2A$^{-}$ NK cells. Since KIR$^{+}$ cells have a much lower proliferative capacity as compared to KIR$^{-}$ ones (Figure 5) (78), NK cells which acquired KIR expression were more or less lost after four weeks of culture. That could be the reason why KIR induction by cytokines has been underestimated in previous studies (78, 95). Nonetheless, when KIR3DL1$^{+}$ NK cells were re-sorted after 12 days of culture in order to minimize the bias of KIR$^{-}$ cell overgrowth, most NK cells maintained KIR expression, similar to *ex vivo* KIR3DL1$^{+}$ ones. Several groups have used murine stromal cells (78) or human fibroblasts (95) in addition to cytokines in order to achieve KIR induction in NK cells. However, as shown in Figure 5, 7 and 8, cytokines alone in the absence of any accessory cells were able to induce KIR expression, but that was restricted to a small subset of NK cells. Interestingly, cells that failed to acquire KIR in a first round of cytokine stimulation were also not able in a second round. Therefore, one could hypothesize that some NK cells already received *in vivo* a signal leading to the initiation of KIR expression and that cytokines would only promote the preferential expansion of this committed subset *in vitro*. However, a recent report from Cichocki et al demonstrated that IL-15 or IL-2 induce c-Myc binding to an upstream distal KIR promoter element which promotes KIR transcription (101), supporting the idea that cytokines are directly required for induction of KIR expression and do not only allow the proliferation or differentiation of a subset of committed NK cells.

Even if it cannot be ruled out that further unknown signals might be required, the results presented in this thesis clearly show that cytokines are essential to induce stable KIR *de novo* expression in a subset of hyporesponsive NK cells.

Next, it was investigated whether cytokine induced KIR acquisition was associated with education of former hyporesponsive NK cells. It could be demonstrated that hyporesponsive NK cells which acquired KIR after cytokine stimulation became cytotoxic competent, suggesting that hyporesponsiveness is not a permanent feature but can be modulated under certain circumstances. Several *in vivo* data provided a hint that hyporesponsive NK cells which do not express inhibitory receptors can still function during infectious diseases (37, 43). Besides, it has been controversially discussed whether IL-2 stimulation could reverse NK cell hyporesponsiveness (39, 78). In the system used in the study presented here, only acquisition of self-MHC specific KIR (i.e. KIR3DL1 in HLA-Bw4 individuals) correlates with induction of cytotoxic competence, while acquisition of non self specific KIR (i.e. KIR3DL1 in HLA-Bw6 individuals) does not. These data show that although KIR *de novo* expression can occur also in the absence of the cognate MHC molecule, in agreement with the stochastic nature of the KIR repertoire (36), acquisition of cytotoxic competence requires the presence of self-MHC. Since cytokine stimulation can confer competence in the absence of any accessory cells, KIR could engage the cognate MHC molecule expressed on the same NK cell (*cis* interaction) or on neighbouring NK cells (*trans* interaction). In mice, it has been recently shown that *cis* interaction between inhibitory Ly49A receptor and MHC class I molecules plays a role for education of NK cells (102). Even if the hypothesis that human NK cell education as well is mediated via *cis* interaction would be very intriguing, there is currently no evidence that the function of KIR is influenced by HLA expression in *cis* (103). The observation that self-MHC molecules expressed on the surface of NK cells can be sufficient to induce NK cell education has important implications in HLA-mismatched hematopoietic stem cell transplantation, where alloreactive NK cells have been shown to be beneficial especially for the treatment of acute myeloid leukemia (104). Since these NK cells would develop and therefore be educated in an KIR-MHC class I mismatched environment it would be of interest, which cells are *in vivo* responsible for educating NK cells. Finally, it was shown that cytokine induced KIR are actually functional, since they are able to inhibit NK cell killing of target cells expressing the cognate MHC ligand, thus suggesting that cytokine induced education should not lead to autoreactivity.

Altogether, the results show that competent NK cells can be generated after cytokine stimulation, thus indicating that hyporesponsiveness does not represent an irreversible state. On the contrary, at least a subset of hyporesponsive NK cells can still be educated to become competent and self tolerant during an immune response. As it was previously shown that KIR acquisition can occur in highly reactive LN (Figure 6), inflamed secondary lymphoid organs might represent one possible peripheral site of NK cell education *in vivo*. This process requires the expression of the cognate MHC ligand and can occur in the absence of any accessory cells.

These results help to clarify the means by which NK cell education is achieved, and might have important implications especially in haploidentical transplantation, where competence of donor alloreactive NK cells reconstituted in MHC mismatched recipients has not been addressed yet.

## 5.3 CD62L expression identifies a subset of polyfunctional CD56$^{dim}$ NK cells

To dissect further the heterogeneity of CD56$^{dim}$ cells, in the third part of this study (chapter 3.3) an extensive *ex vivo* analysis of human NK cells has been performed according to the expression of markers related to differentiation, migration or competence. In particular, CD56$^{dim}$ cells have been further dissected according to the expression of the lymph node homing marker CD62L. Thus, a new subset of polyfunctional NK cells could be identified since it was shown that CD56$^{dim}$ CD62L$^{+}$ NK cells represent the only human NK cell subset endowed with full effector functions after stimulation with cytokines and via activating receptors as well as with high self renewable ability.

It could be demonstrated that both $CD56^{bright}$ and $CD56^{dim}$ $CD62L^{+}$ cells are actively proliferating *in vivo* under steady state conditions as well as in the course of an acute viral infection such as yellow fever infection. *In vivo* proliferation of NK cells under steady state conditions and in the early phase of a viral infection, is largely non-specific and most probably cytokine driven (105, 106). Thus, IL-15 has been shown to be the limiting factor for homeostatic expansion of NK cells (107, 108) and there are evidences that IFN-α/β induced IL-15 is also involved in virus-induced NK cell proliferation (109, 110). Considering the high responsiveness of $CD56^{bright}$ and $CD56^{dim}$ $CD62L^{+}$ NK cells to cytokines, it is likely that $Ki67^{+}$ cells within these NK cell subsets might have been activated *in vivo* by cytokines. The high ability of $CD56^{dim}$ $CD62L^{+}$ cells to respond to γ-chain cytokines was associated to elevated levels of STAT5 phosphorylation. Efficiency of STAT or ERK activation after cytokine stimulation has been correlated to the amounts of SET, a potent inhibitor of protein phosphatase 2A (PP2A) activity (81). Trotta et al. have indeed shown that SET expression is higher in $CD56^{bright}$ than in $CD56^{dim}$ cells and knocking down of SET resulted in decreased STAT5, STAT4 and ERK phosphorylation after cytokine stimulation (81).

Cytokine and DC stimulation resulted also in massive IFN-γ production by $CD56^{bright}$ and $CD56^{dim}$ $CD62L^{+}$ cells while $CD62L^{-}$ cells were very inefficient. $CD56^{dim}$ NK cell ability to proliferate and to produce IFN-γ after DC or cytokine stimulation stringently correlated with CD62L and not with KIR or NKG2A expression. Therefore, the higher proliferative competence of $KIR^{-}$ or $NKG2A^{+}$ cells observed in previous reports (Figure 5) (78), might rather be a consequence of enrichment of $CD62L^{+}$ NK cells in these populations, and not of a direct correlation to one of these markers. Interestingly, among $CD56^{dim}$ $CD62L^{+}$ and $CD62L^{-}$ NK cells, CD27 expression identified NK cells with a higher capacity to produce IFN-γ, showing that the combination of CD62L and CD27 (but not CD27 alone) identifies the most potent IFN-γ producers among $CD56^{dim}$ NK cells. Although $CD56^{bright}$ have been long considered as the cytokine producers, these cells were almost unable to express IFN-γ and TNF after activating receptor stimulation, while $CD56^{dim}$ $CD62L^{+}$ as well as $CD62L^{-}$ cells were conversely very efficient. In line with IFN-γ and TNF expression after activating receptor stimulation, cytotoxicity against MHC class I negative target cells was very poor in $CD56^{bright}$ but comparable in both $CD56^{dim}$ $CD62L^{+}$ and $CD62L^{-}$ cells.

While expression of self-MHC-specific KIR or NKG2A seemed to have no influence on the capacity to proliferate and to produce IFN-γ, NK cells bearing a self-MHC class I specific KIR were more cytotoxic than $KIR^-$ cells with just minor enhancing impact of CD62L or NKG2A, probably reflecting a recent *in vivo* activation by cytokines. Acquisition of KIR clearly represents a crucial step during NK cell differentiation, since cytotoxic cells were mostly included within NK cells expressing self- MHC specific KIR, in line with previous results (39, 40). Concerning NKG2A, it was already reported that its influence on NK cell competence is rather low compared to self-MHC-specific KIR (40). When analysing the impact of NKG2A versus KIR or CD62L expression on cytotoxic competence, it was shown that NKG2A expression per se is almost irrelevant. The reported influence of CD27 expression on cytotoxic capacity of NK cells is difficult to interpret. In mice, it has been shown that $CD27^+$ cells are more cytotoxic whereas in humans $CD56^{dim}$ $CD27^+$ cells were less cytotoxic (66, 111) then $CD27^-$ ones. Both studies did not take into account the influence of self-MHC-specific inhibitory receptors. Since CD27 expression negatively correlates with KIR or Ly49 receptor expression in total NK cells (65, 66, 111), as well as within the $CD56^{dim}$ subset ($p = 0.002$, data not shown), definite conclusions cannot be driven from published results. In order to assess this issue, analysis of cytotoxicity should be performed on $CD27^+$ and $CD27^-$ cells expressing or not self-MHC specific KIR. However, due to the extremely low number of $CD27^+$ $KIR^+$ cells, this experiment is rather challenging. Based on the results obtained in this study, it seems that the ability to proliferate and to produce IFN-γ in response to cytokines is clearly dissociated from the ability to kill and to produce IFN-γ after activating receptor stimulation in most NK cells with the exception of $CD56^{dim}$ $CD62L^+$ cells, which therefore represent a unique polyfunctional subset. $CD56^{dim}$ $CD62L^+$ NK cells are special not only in relation to their functions but also to their homing potential. Indeed, the data presented here suggest that a fraction of $CD56^{dim}$ $CD62L^+$ NK cells would has the potential to migrate through high endothelial venules and to patrol secondary lymphoid tissues, similar to what has been shown for central memory T cells (112). Due to their homing potential as well as functional properties, $CD56^{dim}$ $CD62L^+$ cells indeed resemble central memory T cells. Recent studies suggested that NK cells primed during viral infections or by cytokines may exhibit memory-like properties during a recall viral response or *ex vivo* restimulation (63, 64). It is exciting to speculate that the $CD56^{dim}$ $CD62L^+$ subset might be enriched in cells that received priming *in vivo.* Hence, these cells might be able to respond rapidly after restimulation in a memory like fashion and further develop into terminally differentiated effectors. Although no data could be provided sustaining this hypothesis, the analysis of telomere length at least sup-

ports the idea that $CD56^{dim}$ $CD62L^{+}$ $KIR^{-}$ NK cells may represent an intermediate step of maturation between $CD56^{bright}$ and $CD56^{dim}$ $CD62L^{-}$ $KIR^{+}$ ones, as also suggested by their phenotype and functions. The finding that differences in telomere length within $CD56^{dim}$ subsets became detectable only when comparing $CD56^{dim}$ $CD62L^{-}$ $KIR^{+}$ with $CD62L^{+}$ $KIR^{-}$ cells, give rise to different interpretations. The favoured explanation is that, among the $CD62L^{-}$ subset, cells that acquired KIR expression displayed the longest proliferative history, and thus differences in telomere length became detectable. Since loss of CD62L and acquisition of KIR expression appear to be independent events, $CD56^{dim}$ $CD62L^{+}$ $KIR^{+}$ or $CD62L^{-}$ $KIR^{-}$ cells also exist, but differences in their telomere length may not be substantial. However, since under selected stimuli CD62L can be up regulated (Figure 3), $CD62L^{+}$ $KIR^{+}$ cells may also include a contamination of “revertants”, which have reacquired CD62L expression. The idea that $CD56^{dim}$ $CD62L^{-}$ cells may be enriched in terminally differentiated cells fits also to the observation that those cells accumulate with age, while frequencies of $CD56^{bright}$ and $CD56^{dim}$ $CD62L^{+}$ cells decrease. Absolute NK cell numbers tend to be maintained or to increase with age (113, 114), and the accumulation of NK cells seems to be a consequence of an increase in the mature $CD56^{dim}$ subset rather than in the less mature $CD56^{bright}$ one (84). The longer infectious history of aged people could result in repeatedly stimulation and expansion of $CD56^{dim}$ $CD62L^{+}$ NK cells accompanied by a progressive differentiation towards $CD56^{dim}$ $CD62L^{-}$ $CD27^{-}$ $KIR^{+}$ NK cells going over several intermediate stages detectable in the peripheral NK cell pool. The concept is also in line with results obtained in a recent study describing NK cell reconstitution in patients undergoing hematopoietic stem cell transplantation (HSCT) (115). Indeed, most of the NK cells derived from PB of patients at day 30 after HSCT were $CD56^{bright}$ as previously shown (56), and almost all $CD56^{dim}$ NK cells expressed CD62L. Percentages of $CD56^{dim}$ $CD62L^{+}$ NK cells progressively decreased with time and became comparable to those of healthy blood donors only around 100 days after HSCT (115).

Based on all these results as well as on many previous observations (53), it can be presumed that early $CD56^{bright}$ NK cells display high ability to respond to DC derived cytokines, which represent the main activating signal in secondary lymphoid organs where $CD56^{bright}$ NK cells reside, but exhibit poor effector functions after activating receptor stimulation. Cytokines provided by DC such as IL-15 (62), IL-18 (60) or IL-12 (61) could be at least one important factor responsible for NK cell priming and NK cell differentiation into $CD56^{dim}$. After restimulation, NK cells would progressively lose their ability to respond to cytokines but would acquire the capacity to display effector functions upon activating receptor stimulation. This property is indeed crucial for a prompt response against infected cells in inflamed tissues. These events would be accompanied by progressive loss of CD62L, CCR7 and CD27 and acquisition of KIR and CXCR1, as also supported by several other studies (57, 66, 77, 78, 96, 116). In this scenario, $CD56^{dim}$ $CD62L^{-}$ $KIR^{+}$ ($CD27^{-}$) cells might correspond to terminally differentiated effector NK cells, while $CD56^{dim}$ $CD62L^{+}$ NK cells may represent cells that patrol secondary lymphoid organs and, when restimulated, display direct effector functions and give rise to terminally differentiated effectors.

The results presented in the third part of this thesis provide further insights into differentiation history of human NK cells and identifies a unique subset of polyfunctional NK cells. Due to their versatility, $CD56^{dim}$ $CD62L^{+}$ NK cells might be important in the defence against pathogens or as immune correlates of protection in vaccination, as it has been shown for polyfunctional T cells (117-119). Moreover, this subset could be an optimal candidate for adoptive NK cell therapy that has been shown to be a feasible strategy to improve cancer therapy in combination or not with HSCT (104, 120).

Overall, this thesis provides new insights into terminal human NK cell differentiation that goes via several intermediate stages which are characterized by distinct functional properties.

# 6 Summary

Human NK cells comprise two main subsets, $CD56^{br}$ and $CD56^{dim}$ cells. In this study, an extensive analysis of human NK cell phenotype and functional characteristics has been performed in order to investigate the developmental relation between NK cell subsets, to elucidate how NK cell competence is acquired and to further dissect the heterogeneity of the $CD56^{dim}$ subset with regard to functions and differentiation history of human NK cells.

It could be shown that upon cytokine activation, $CD56^{br}$ differentiate into $CD56^{dim}$ NK cells and that this process might take place in inflamed secondary lymphoid organs (SLO). One of the crucial markers acquired during this process is KIR, the main MHC-specific inhibitory receptors responsible for self versus non self recognition. Previously, it has been shown that only cells expressing self-MHC specific KIRs are responsive to activating stimuli. In this study, it was demonstrated that induction of self-MHC specific KIR by cytokines leads to acquisition of functional competence. *Ex vivo* analysis of human tissues suggests that acquisition of KIR and consequently of cytotoxic competence may occur in inflamed SLO. Finally, it was demonstrated that $CD56^{dim}$ NK cells do not represent a homogenous population. When dissected for CD62L and KIR expression, a new subset of NK cells could be identified, namely $CD56^{dim}CD62L^{+}$, which uniquely combines properties of $CD56^{br}$ NK cells, particularly high IFN-γ production upon cytokine stimulation, proliferation and potential to migrate into SLO, with the capacity of $CD56^{dim}$ to kill, produce cytokines upon activating receptor stimulation and to migrate into inflamed tissues. *Ex vivo* analysis of the function, phenotype, telomere length and frequencies during ageing of $CD56^{br}$, $CD56^{dim}CD62L^{+}$ and $CD56^{dim}CD62L^{-}$ NK cells suggest that $CD56^{dim}CD62L^{+}$ cells represent an intermediate stage of NK cell maturation between the more immature $CD56^{br}$ and the terminally differentiated $CD56^{dim}$ $CD62L^{-}$ NK cells.

# 7 Zusammenfassung

Humane NK Zellen können in $CD56^{br}$ und $CD56^{dim}$ NK Zellen unterteilt werden. In dieser Arbeit wurde untersucht, in welchem Zusammenhang die verschiedenen NK Zell Populationen stehen und wie funktional kompetente NK Zellen generiert werden. Des Weiteren wurde die Heterogenität der $CD56^{dim}$ NK Zell Population in Bezug auf Funktionalität und Differenzierungsstadien analysiert.

Es konnte gezeigt werden, dass $CD56^{br}$ NK Zellen in $CD56^{dim}$ NK Zellen differenzieren. Währenddessen werden u.a. MHC-I spezifische inhibierende Rezeptoren (KIR) erworben. Diese sind essentiell für die Unterscheidung zwischen “Selbst” und “Nicht-Selbst”, wobei nur NK Zellen, die Selbst-MHC-spezifische KIRs tragen, funktional kompetent sind. In der vorliegenden Arbeit konnte darüber hinaus gezeigt werden, dass zuvor anerge NK Zellen nach Zytokin-induzierter Expression eines Selbst-MHC-spezifischen KIRs kompetent werden. *Ex vivo* Analysen humaner Gewebe lassen vermuten, dass diese Prozesse während einer Entzündung in sekundären lymphatischen Organen (SLO) stattfinden könnten.

Auch $CD56^{dim}$ NK Zellen selbst sind nicht homogen, hingegen können anhand der Expression von KIRs oder CD62L, welches für die Migration in SLO wichtig ist, weitere Subpopulationen unterschieden werden. Eine umfassende Analyse bezüglich KIR und CD62L Expression führte zur Identifizierung einer zuvor nicht charakterisierten $CD56^{dim}CD62L^{+}$ NK Zell Population, welche die Fähigkeiten von $CD56^{br}$, Zytokine zu produzieren und zu proliferieren, mit einem hohen zytotoxischen Potenzial, vereinigt.

Weitere *ex vivo* Untersuchungen des Phänotyps, der Telomerlängen und der Verteilung in Relation zum Alter lassen vermuten, dass die Differenzierung humaner NK Zellen von $CD56^{br}$ über $CD56^{dim}CD62L^{+}$ zu $CD56^{dim}CD62L^{-}$ verläuft, wobei die Zellen mit fortschreitender Differenzierung ihre Fähigkeit auf Zytokine zu antworten verlieren und dafür die Fähigkeit erlangen, über aktivierende Rezeptoren stimuliert zu werden.

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# 9 Appendix

# I Abbreviations

| | |
|---|---|
| α | anti |
| ADCC | antibody dependent cellular cytotoxicity |
| APC | antigen presenting cell |
| BCR | B cell receptor |
| BSA | bovine serum albumin |
| BM | bone marrow |
| BrefA | Brefeldin A |
| CD | cluster of differentiation |
| CFDA | Carboxyfluoresceindiacetat |
| CFSE | Carboxzfluoresceinsuccimidylester |
| CTL | cytotoxic T lymphocyte |
| DC | dendritic cell |
| EDTA | ethylene diaminetetraacetic acid |
| ERK | extracellular signal-regulated kinase |
| FACS | fluorescence activated cell sorting |
| FcγRIII | constant fraction γ receptor III |
| FCS | forward scattered light |
| g | Relative centrifuge force ($g = 9.81\ m/s^2$) |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| HLA | human leukocyte antigen |
| HPC | hematopoietic progenitor cell |
| IFN-γ | interferon γ |
| IL | interleukin |
| ITIM | immunoreceptor tyrosine-based inhibitory motif |

| | |
|---|---|
| KIR | killer immunoglobuline-like receptor |
| LN | lymph node |
| LPS | lipopolysaccharide |
| mAb | monoclonal antibody |
| MACS | magnetic activated cell separation |
| MHC | major histocompatibility complex |
| MFI | mean fluorescence intensity |
| Monokine | monocyte-derived cytokine |
| mDC | myeloid DC |
| NCR | natural cytotoxicity receptors |
| NK | natural killer |
| NKR | NK receptor |
| PAMP | pathogen associated molecular pattern |
| PB | peripheral blood |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PI | propidium iodide |
| RPMI | Roswell Park Memorial Institute |
| RTL | relative telomere length |
| ORF | open reading frame |
| SA | Streptavidine |
| SLO | secondary lymphoid organs |
| SSC | side scatter |
| STAT | Signal Transducers and Activator of Transcription |
| TCR | T cell receptor |
| $T_H$ | T helper |
| TLR | Toll-like receptor |

| | |
|---|---|
| TNF | tumour necrosis factor |
| YFV | yellow fever virus |

## II Eidesstattliche Erklärung

Hiermit bestätige ich, Kerstin Jülke, geboren am 12.06.1974 in Bernau, dass ich die vorliegende Dissertation selbständig angefertigt habe.

Ich versichere, ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen zu haben.

Desweiteren versichere ich, dass die vorliegende Arbeit nie in dieser oder anderer Form Gegenstand eines früheren Promotionsverfahrens war.

Berlin, den

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